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CLUSTER MODEL OF CROSSING OVER

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Received March 21, 1960

MANY models of crossing over can be constructed that will fit more or less well with observed data of recombination frequencies and quadruplet frequencies. The model examined here is worthy of attention because it is a simple and attractive explanation of recent data of undoubted significance. When intervals which have a very low frequency of crossing over are studied, the frequency of multiple crossovers is too high. There is negative interference (PRITCHARD 1955).

To account for this, PRITCHARD proposed that crossovers occur in tight clusters. We can envisage the number of crossovers in a cluster to be constant for a species or for a chromosome. Map distance would be a function of the frequency of clusters.

The model: The frequency distribution of crossovers within a tight cluster will be taken to be a Poisson distribution with a mean of m_2, and the clusters themselves will be taken, in the first instance, to be in a Poisson distribution with a mean of m_1. This type of distribution (where a primary event occurs in a Poisson distribution and these primary events are themselves distributed in a Poisson) has been examined in several contexts where the primary and secondary events are respectively the number of passengers injured per automobile accident and the number of automobile accidents, the number of neutrons produced by an atomic collision and the number of collisions and, notably, the number of eggs laid by an insect and the number of layings. The latter case was examined by GEOFFREY BEALL (1940) to whom I am indebted for the argument leading to the present application of this so-called contagious distribution.

Distribution of tetratype and other frequencies: It has been shown (PAPAZIAN 1951) that if crossovers occur in a Poisson distribution then the frequency of tetratype quads equals $2/3\ (1 - e^{-3/2m})$----(1) where m is the mean frequency of crossovers. In extending this to the contagious distribution let the mean number of crossovers within a cluster be m_2, and let the mean number of clusters be m_1. In case there are S clusters the expected number of crossovers from all is Poisson with a mean Sm_2. In this case we should, from equation (1), get a proportion of $2/3\ (1 - e^{-3/2Sm_2})$ tetratypes. The chance of there being S tight groups is, now, $e^{-m_1}\frac{{m_1}^S}{S!}$. This makes a total contribution of $e^{-m_1}\frac{{m_1}^S}{S!}\,2/3\ (1 - e^{-3/2Sm_2})$. We can

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accordingly say that the over-all proportion of tetratypes, T, is:

$$\sum_{S=0}^{\infty} e^{-m} \frac{m_1^S}{S!} \frac{2}{3} \left(1 - e^{-\frac{3}{2} S m_2}\right)$$

$$\text{or} \quad T = \frac{2}{3}\left(1 - e^{-m_1\left(1 - e^{-\frac{3}{2}m_2}\right)}\right) \text{-----(2)}^2$$

The frequency of nonparental ditype quads, N, in the Poisson distribution of crossover model, has been shown to be $N = 1/2 - 1/3\ (1 - e^{-3m/2}) - (e^{-m})/2$ (PAPAZIAN 1952) where m is the mean frequency of crossovers.

By rearrangement: $N = 1/6 + 1/3\, e^{-3/2m} - 1/2\, e^{-m}$ and according to the cluster model, by an argument similar to that used for tetratype frequencies,

$$N = \sum_{S=0}^{\infty} e^{-m_1} \frac{m_1^S}{S!} \left(\frac{1}{6} + \frac{1}{3} e^{-\frac{3}{2} m_2 S} - \frac{1}{2} e^{-m_2 S}\right)$$

$$= \frac{1}{6} e^{-m_1} \sum_{S=0}^{\infty} \frac{m_1^S}{S!} + \frac{1}{3} e^{-m_1} \sum_{S=0}^{\infty} e^{-\frac{3}{2} m_2 S} \frac{m_1^S}{S!} - \frac{1}{2} e^{-m_1} \sum_{S=0}^{\infty} e^{-m_2 S} \frac{m_1^S}{S!}$$

$$= \frac{1}{6}\left(1 + \frac{m_1}{1!} + \frac{m_1^2}{2!} + \cdots\right) + \frac{1}{3} e^{-m_1}\left(1 + m_1 e^{-\frac{3}{2} m_2} + \frac{\left(m_1 e^{-\frac{3}{2} m_2}\right)^2}{2!} + \cdots\right)$$

$$- \frac{1}{2} e^{-m_1}\left(1 + m_1 e^{-m_2} + \frac{\left(m_1 e^{-m_2}\right)^2}{2!} + \cdots\right)$$

$$= \frac{1}{6} + \frac{1}{3}\left(e^{-m_1}\ e^{m_1 e^{-\frac{3}{2} m_2}}\right) - \frac{1}{2}\left(e^{-m_1}\ e^{m_1 e^{-m_2}}\right)$$

$$N = \frac{1}{6} + \frac{1}{3}\left(e^{-m_1\left(1 - e^{-\frac{3}{2} m_2}\right)}\right) - \frac{1}{2}\left(e^{-m_1\left(1 - e^{-m_2}\right)}\right) \text{-----(3)}$$

and by a similar argument, from $P = 1/2 - 1/3\ (1 - e^{-3/2m}) + 1/2\, e^{-m}$ (PAPAZIAN 1952) we get

$$P = 1/6 + 1/3\ \left(e^{-m_1\left(1 - e^{-3/2 m_2}\right)}\right)\ 1/2\ \left(e^{-m_1\left(1 - e^{-m_2}\right)}\right) \text{————(4)}$$

where P is the frequency of parental ditype quads.

And from $R = 1/2\ (1 - e^{-m})$ (HALDANE 1919) we get

$$R = 1/2\ \left(1 - e^{-m_1\left(1 - e^{-m_2}\right)}\right) \text{————(5)}$$

Estimation of parameters: The cluster model requires two parameters, m_1 and m_2. If m_1 is a function of map distance and m_2 is a constant, we must examine how available or procurable data can be used to estimate m_2 and also to discriminate between a cluster model and a simple Poisson model.

[2] This formula is published by kind permission of GEOFFREY BEALL of the Gillette Safety Razor Blade Company, and of the Gillette Safety Razor Blade Co.

The making of genetic maps from recombination data will not be altered by a cluster theory inasmuch as the addition law still holds, for if m_2 is constant and $c = 1 - e^{-m_2}$ then from (5) $R = \frac{1}{2}(1 - e^{-m_1 c})$

$$m_1 c = \ln \frac{1}{1 - 2R} \quad \text{and if} \quad m_{1(12)} = m_{1(1)} + m_{1(2)}$$

$$\text{then } \frac{1}{c} \ln \frac{1}{1 - 2R_{12}} = \frac{1}{c} \ln \frac{1}{1 - 2R_1} + \frac{1}{c} \frac{1}{1 - 2R_2}$$

$$\text{or } R_{12} = R_1 + R_2 - 2R_1 R_2$$

Similarly, for the addition of tetratype, or second division segregation, frequencies, from (2):

$$\frac{1}{c} \ln \frac{1}{1 - 3/2T_{12}} = \frac{1}{c} \ln \frac{1}{1 - 3/2T_1} + \frac{1}{c} \ln \frac{1}{1 - 3/2T_2}$$

$$\text{or } T_{12} = T_1 + T_2 - 3/2 \; T_1 T_2$$

which is the same as for the simple Poisson model (PAPAZIAN 1952). There remain three different kinds of data which can discriminate between the two models and also provide an estimate for m_2. The distribution of multiple crossovers when a very short interval is involved, the relation between tetratype and nonparental ditype frequencies and the frequency of chiasmata between two points where the frequency of crossing over is known.

Multiple crossovers in short regions: The data which suggested to PRITCHARD that crossovers occurred in clusters consisted of progeny which were selected for crossovers between two adenineless mutants in *Aspergillus nidulans*. The frequency of crossing over between the two *ad* mutants was very small, of the order of 10^{-5}. Among progeny from crosses between *ad8* and *ad10* (PRITCHARD's Table 6) and between *ad8 and ad12* (PRITCHARD's Table 5) which had a crossover in this narrow region, 116 plants did not have crossovers on either side of the *ad* loci, 47 plants had a crossover on one or the other side and four plants had a crossover on both sides.

If we consider that in this experiment a single crossover of a cluster has been isolated, and other members of the same cluster are represented by crossovers on either side of *ad*, then these frequencies can give an estimate of m_2 for clusters involving *ad*.

Estimation is complicated by the fact that we do not know the zero term for the Poisson series. If crossovers within a cluster occur in a Poisson distribution, then sometimes there will be effective pairing; there will be a cluster so to speak, but it will contain no crossovers. The maximum likelihood estimation of the mean of a truncated Poisson has been examined by DAVID and JOHNSON (1952) in

connection with the mean number of factory workers injured where the number of uninjured was unknown.

In the present case, the numbers of single, double, and triple crossovers is 116, 47 and four with a total, excluding zeros, of 167. The mean of the truncated Poisson is then $\frac{116\ 2\times 47\ 3\times 4}{167} = 1.329$. The probability of each term of a truncated Poisson is $P_x = \frac{e^{-m}}{1\text{-}e^{-m}} \frac{mX}{X!}$ where m is the mean. The maximum likelihood estimate of m is given by $\frac{m}{1\text{-}e^{-m}} = \bar{X} = T/N$ where T is the total and N the number of successful trials in a truncated Poisson. In the present case, $\frac{m}{1\text{-}e^{-m}}$ $= 1.329$, and this can be solved for m easily by constructing a graph from the table in DAVID and JOHNSON (1952). By this method $m = 0.6$ which means this data gives an estimate of 0.6 for m_2 or the number of crossovers within a cluster. From this estimate of the mean, the expected numbers of zeros, singles, doubles, etc., crossovers can be calculated. The expected and observed numbers are:

	Single	Double	Triple and more
expected	121.98	36.59	8.43
observed	116	47	4

A χ^2 goodness of fit test on these figures with one degree of freedom gives P $\sim$ 2.1 percent.

Relation of N *and* T *frequencies:* On a simple Poisson model the relation between the frequencies of tetratypes and nonparental ditypes is given by $N = 1/2\ (1\text{-}T\text{-}(1\text{-}3/2T)^{2/3})$ (PAPAZIAN 1952); this relationship is shown by the bottom curve in Figure 1.

On a cluster model, from (2) $1/3e^{-m_1(1-e^{-3/2m_2})} = 1/3 - 1/2T$. If we let $c = 1/3 - 1/2T$ then, from (3), $N = 1/6\ c - 1/2^{e\text{-}m_1(1-e-m_2)}$ and $c = N - 1/6\ 1/2e\text{-}m_1{}^{(1-e\text{-}m_2)}$ $= 1/3 - 1/2T$ from which $N = 1/2(1\text{-}T\text{-}e^{-m_1(1-e^{-m_2})})$ ..... (6)

Curves showing this relationship when $m_2 = 0.5$, 1, 3, and ∞ are given in Figure 1. These curves were constructed for each value of m_2 by calculating T for appropriate values of m_1 in (2) then substituting these corresponding values of m_1 and T in (6) to get the corresponding value of N.

The algebraic solution of the expression of N as a function of T is heavy. As regards the recombination frequency, an algebraic solution is feasible and gives

$$R = -1/2\ (1\text{-}3/2T)^{\frac{(1-e^{-m_2})}{(1-e^{-3/2m_2})}} + 1/2.$$

Chiasmata and crossing over: If a chiasma, visible in tetrads, corresponds to regions where a cluster of crossovers occurs then m_1 should correspond to the frequency of chiasmata. From (2)

$$m_1 = \frac{ln\frac{1}{(1\text{-}3/2)T}}{1\text{-}e^{-3/2m_2}} \text{ and from (5) } m_1 = \frac{ln(1/2)R}{1\text{-}e^{-m_2}} \text{ which gives the relationship}$$

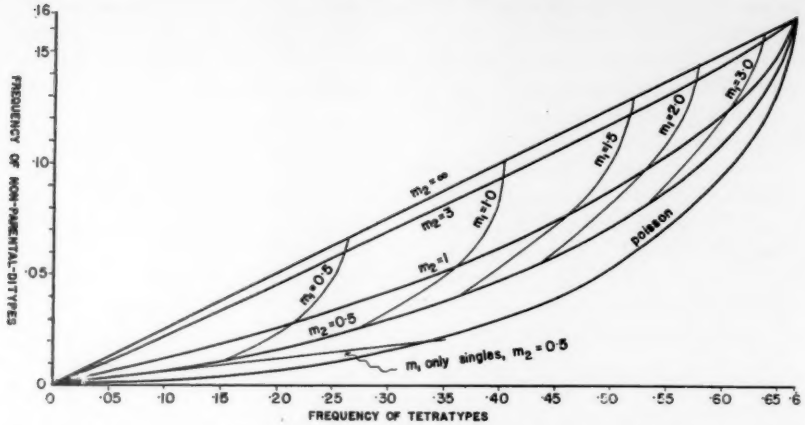

FIGURE 1.—Theoretical relationships between tetratype and nonparental ditype frequencies. Top four long horizontal curves give T and N for four values of m_2 on a cluster model, bottom curve on a simple Poisson model. Five, more vertical, shorter curves are isograms giving positions of five m_1 values on the cluster model curves. The short straight curve represents the cluster model with complete interference between clusters, only single clusters occurring.

between chiasmata and tetratype frequencies and chiasmata and recombination frequencies, respectively.

In Figure 1 the short, more nearly vertical, curves, give N and T values for values of m_1 of 0.5, 1.0, 1.5, 2.0 and 3.0. Where one of these five curves crosses a long curve for a certain m_2 value, the T and N value along each axis give T and N frequencies for that value of m_1 or chiasma frequency and m_2. Since $R = N + \frac{1}{2}T$, R values can be read from these curves.

DISCUSSION

This study of the cluster model has neglected the troublesome consideration of the extent of the cluster. In the derivation of (2), (3), (4) and (5) the crossovers are assumed to occur all at a point whereas in PRITCHARD's data the cluster has a definite but unknown extent on either side of the *ad* complex.

As regards the fitting of data to this model: the calculated numbers of multiple crossovers are not outrageously different from PRITCHARD's observations. The deviations are in the direction of not enough singles and triples, too many doubles. No plausible explanation of this occurs to the author. Data from STRICKLAND (1958) in which quads were analyzed give N and T values which fall below the $m_2 = 0.5$ curve in Figure 1.

From his Table 2, $T = \frac{156}{392} = 0.398$, and $N = \frac{5}{392} = 0.013$; and from his Table 4, $T = \frac{203}{574} = .428$, and $N = \frac{21}{574} = .037$. If m_2 is constant for an organism then for *A. nidulans* it should be around 0.5 from PRITCHARD's multiple crossover data.

The cluster model can accommodate discrepancies of data from more widely separated loci such as STRICKLAND's, by allowing positive interference between clusters. If such interference is complete, then $T = m_1\, 2/3\ (1\text{-}e^{-3/2m_2})$, and $N = m_1\ (1/6 + 1/3e^{-3/2m_2} - 1/2e^{-m_2})$; the items in brackets being constants, the relationship is linear. This curve for $m_2 = 0.5$ is drawn in Figure 1. It will be noticed that in this case, T can never rise above 0.36 and N never above 0.022, instead of 2/3 and 1/6 which is true for all other curves shown in Figure 1.

There are few studies on the relationship between chiasmata and crossing over. Those of DARLINGTON (1934) on corn deviate in the direction of the crossover value being too low. This deviation may, and has, been diminished by more complete genetic data but can be also explained by a cluster theory of crossing over because you would sometimes get a cluster with zero crossovers. Of course whether such zero clusters would show up as chiasma is a moot point.

SUMMARY

The consequences of a theory of crossing over in which crossovers occur in tight clusters is examined. Formulas relating recombination, quadruplet, and chiasmata frequencies are developed and parameters roughly estimated from published data.

ADDENDUM

I would like to draw attention to a paper by R. H. PRITCHARD (Localized negative interference and its bearing on models of gene recombination. Genetical Research **1**: 1–24) which appeared after this went to press.

PRITCHARD calculates the mean number of crossovers per cluster or effective pairing region using new data and a different approach. His approach makes use of the concept and the value of the length of an effective pairing region but in general the assumptions in PRITCHARD's paper appear to be the same as those made here, notably a Poisson distribution of crossovers within a cluster. I believe PRITCHARD's model, as used to calculate the mean crossovers per effective pairing region, is essentially equivalent to that used here.

PRITCHARD arrives at the value of 0.6 crossovers per effective pairing region which is the same as that found above. The exact coincidence of values must be regarded as coincidental.

ACKNOWLEDGMENT

I wish to thank DR. ALAN JAMES of the Department of Mathematics, Yale University for guidance in dealing with the truncated Poisson.

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RELATION BETWEEN THE PIGMENTATION AND PTERIN DERIVATIVES OF CHROMATOPHORES DURING DEVELOPMENT IN THE NORMAL BLACK AND TRANSPARENT SCALED TYPES OF GOLDFISH (CARASSIUS AURATUS)[1]

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FLUORESCENT substances of pterin type have been known to exist abundantly in the colored portions of the adult skin of Amphibia and Pisces, while they can scarcely be found in th ventral white portions. These substances were shown to be greatly accumulated in the chromatophores of bullfrogs (HAMA and OBIKA 1959). In Cyprinidae, the violet fluorescent pterins are the main components as reported by ZIEGLER-GÜNDER (1956). The chemical nature of these substances has been studied by several investigators (POLONOVSKI, BUSNEL and PESSON 1943; HÜTTEL and SPRENGLING 1943; TSCHESCHE and KORTE 1951, 1953; HIRATA, NAWA, MATSUURA and KAKIZAWA 1952; KORTE 1954; MATSUURA, NAWA, GOTO and HIRATA 1955; NAWA, MATSUURA, GOTO and HIRATA 1955; ZIEGLER-GÜNDER 1956; TSCHESCHE and GLASER 1958). Our reports on Cyprino-pourpre A1, Cyprino-pourpre A2 and Cyprino-pourpre B, which were found in the adult skin and scales in carp and normal red scaled goldfish (Ryûkin), are relevant to this. We showed that these substances corresponded to isoxanthopterin, 7-hydroxybiopterin and isoxanthopterin-6-carboxylic acid, respectively. Besides these substances, another violet fluorescent substance of pterin type, Cyprino-pourpre C, has been found in their larvae. All of these substances appear approximately in accordance with the first pigmentation of chromatophores such as melanophores or xanthophores in the course of development (HAMA, GOTO and KUSHIBIKI 1952; KUSHIBIKI, HAMA and GOTO 1954; MORI and MATSUMOTO 1956, 1957). These facts suggest a close relationship between the pigmentation and pterin in chromatophores. To clarify further such relationship, it is desirable to employ different types of one species which differ in the mode of pigmentation. For such a purpose, the normal black and the transparent scaled types of goldfish, which differ genetically in their pigmentation, were used. This paper deals with the difference between these two types in the degree of pigmentation and in pterin contents during development.

MATERIALS AND METHODS

The materials used are the offspring by the inbreeding of homozygous normal black scaled fish and of homozygous transparent scaled fish, respectively. Homo-

[1]Contribution No. 19 from Biological Laboratory, Keio University.

zygous fish have been segregated by self-breeding heterozygous transparent scaled fish "Sanshiki-démékin" (Calico telescope-eyed goldfish).

Normal development of chromatophores in the two different types of goldfish

Homozygous normal black scaled type (tt): At room temperature of about 21°C, the eye cup becomes pigmented 36 (33–40) hours after spawning. In about ten hours, melanophores appear along the ventral border of myotomes and blood vessels, then in the head and trunk regions. After this time, the melanophores increase in number and extend over all of the surface of the body. After the appearance of melanophores, guanophores appear first on the eye. Subsequently, xanthophores appear, at first on the dorsal surface of the head, shortly before hatching. About 3.5–4.0 days after spawning, the embryo begins to hatch out. Thus, the larva assumes a gray or brownish colored appearance. About 40 days after hatching, the larva is transformed into adult form, being colored a velvet black. It is in this stage that "Ryûkin" assumes a red colored appearance as a result of depigmentation of larval melanophores. The depigmentation of larval melanophores, as such, is presumed to occur in the normal black scaled type fish in which the shape of adult melanophores is quite different from that of larval ones. In the adult, the melanophores and xanthophores become distributed richly on the dorsal side with fewer numbers towards the ventral side where they are generally lacking. The guanophores are most prominent on the ventral surface where they give a dead white appearance to the abdomen.

Homozygous transparent scaled type (TT): Until about ten days after hatching, when the larva reached 6 to 7 mm in length, pigmentation of this type cannot be clearly distinguished from the *tt* type, the chromatophores differentiating to the same degree in both types. After ten days, both types can be distinguished through their different colors, the precocious depigmentation taking place in the transparent scaled type fish. First of all, the xanthophores begin to depigment along the dorsal region, and their depigmentation extends antero-posteriorly until all xanthophores completely disappear within a few days. The larva then becomes gray in color. Shortly after the beginning of depigmentation of xanthophores, the guanophores in the eye begin to disappear. Almost at the same time as the blackening of the eyeball, the guanophores of peritonium also disappear. Finally, the melanophores, which continued to increase in number at a slow rate (compared with the *tt* type) until about 12–13 days after hatching, begin to depigment, and their melanin granules disappear from the entire surface in a few days. Thus, the larva becomes quite transparent.

Chemical nature of pterins found in goldfish

Various names were proposed in our previous papers for the pterins isolated from the larvae and adult skins of frog and fish by the use of paper chromatography. Some of these have been clarified in their chemical nature (HAMA 1953; HAMA and GOTO 1954, 1955; HAMA, GOTO and KUSHIBIKI 1954; KUSHIBIKI *et al.* 1954; MORI and MATSUMOTO 1956, 1957; FIDLER and WOOD 1957; TSCHESCHE and GLASER 1958; GOTO and HAMA 1958; HAMA and OBIKA 1958; FORREST, HAT-

FIELD and VAN BAALEN 1959; HAMA, GOTO, OBIKA and MATSUMOTO 1959; VISCONTINI and MÖHLMANN 1959; HAMA, MATSUMOTO and MORI 1960; MORI, MATSUMOTO and HAMA, unpublished data). The nomenclatures, chemical natures and mutual relations of fluorescent substances found in the goldfish are given in Tables 1 and 2 and in Figure 1. Among these substances, the violet fluorescent substances are the characteristic and main components in Cyprinidae as described before. These data also confirm that there is no difference in the natures of respective pterins among the normal black and red scaled types and in carp. The detail will be published in a forthcoming paper.

TABLE 1

Chemical natures of the fluorescent substances appearing in goldfish

Nomenclature	Chemical structure	Color of fluorescence
Cyprino-pourpre A1 (Rana-chrome 4)	isoxanthopterin (2-amino-4, 7-dihydroxypteridine)	violet
Cyprino-pourpre A2	7-hydroxybiopterin (Ichthyopterin)	violet
Cyprino-pourpre B	isoxanthopterin-6-carboxylic acid	violet
Cyprino-pourpre C	?	violet
Cyprino-rouge	?	red
Rana-chrome 1	biopterin (2-amino-4-hydroxy-6-(1′, 2′-dihydroxypropyl)-pteridine)	blue
Rana-chrome 3	?	blue
Rana-chrome 5	2-amino-4-hydroxy-6-carboxypteridine	blue
Xanthopterin	2-amino-4, 6-dihydroxypteridine	bluish green
Rhacophoro-jaune	2-amino-4, 6-dihydroxy-6-lactyl-6, 7-dihydropteridine*	yellow
Riboflavin		yellow
FMN	flavin mononucleotide	yellow
FAD	flavinadenine dinucleotide	yellow

* Postulated formula proposed by FORREST *et al.* (1959). See also FIDLER and WOOD (1957) and VISCONTINI and MÖHLMANN (1959).

TABLE 2

Maximum wavelength in ultraviolet absorption and fluorescence spectra of violet fluorescent pterins found in goldfish

	Ultraviolet absorption spectra				Fluorescence spectra	
	0.1N NaOH		0.1N HCl		McIlvaine's buffer *p*H 5.0	
	Max. 1 (mμ)	Max. 2	Max. 1 (mμ)	Max. 2	Beckman type (mμ)	Farrand type (mμ)
7-Hydroxybiopterin (Cyprino-pourpre A2)	256	342.5	289	344	416	400
Isoxanthopterin (Cyprino-pourpre A1)	253	339	285	340	410	387
Cyprino-pourpre C	258	339	285	343	415	397

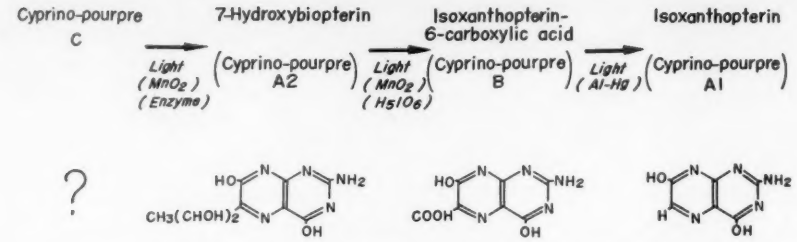

FIGURE 1.—Mutual chemical relation among violet fluorescent pterins found in goldfish.

Extraction of fluorescent substances

Extraction was carried out on the whole egg, embryo and larva and on the skins and scales in the adult. In the embryo and larva, pterins are found abundantly in the skin part. The materials of respective stages were washed carefully with redistilled water, blotted and weighed. The fluorescent substances were obtained by the following methods. (1) Extraction with trichloroacetic acid: The materials were homogenized in a volume of ten percent trichloroacetic acid equal to 20 times the fresh weight. The homogenate was heated thrice in a water bath for 15 minutes at 70°C and then, after cooling, centrifuged at 3000 rpm for five minutes. The combined supernatant fluid was washed six times with one half the volume of ethyl ether to remove the trichloroacetic acid. The ether layer, separated by centrifugation, was discarded at each washing. The resulting aqueous extracts were reduced to about 1.0 or 0.5 ml over concentrated H_2SO_4 under lower pressure. (2) Extraction with ethanol: The extraction was carried out following a slight modification of the method of AWAPARA (1948). This method used originally to extract free amino acids is suitable for obtaining easily extractable or unstable pterins, e.g., Cyprino-pourpre C. The materials were homogenized in a volume of absolute ethanol equal to 20 times the fresh weight. To the homogenate, redistilled water was added to a concentration of 70 percent. This ethanol solution was heated thrice in a water bath for 15 minutes at 80°C, then centrifuged. Chloroform, equal to three times the resulting supernatant fluid, was added to remove the protein and fat soluble substances. The aqueous solution containing the fluorescent substances, which was separated by centrifugation after vigorous shaking, were reduced to about 0.5 ml *in vacuo*.

Fluorimetry

Among the main components of pterins in goldfish, isoxanthopterin-6-carboxylic acid is found in very small amounts so that the quantitative estimation was restricted to 7-hydroxybiopterin (Ichthyopterin), isoxanthopterin and Cyprino-pourpre C. The fluorimetric estimation (MATSUMOTO and MORI 1958) was carried out on the materials of respective developmental stages. The individuals in the process of depigmentation were variable in growth so that exceptionally large or small ones were avoided. The fluorescent substances extracted by method

(1) were purified by paper chromatography. The concentrated extracts were developed twice by *n*-propanol-1% NH_4OH (2:1) by the ascending method and dried by aeration in the dark room. The substances were eluted with redistilled water, from the respective homogenous band of paper, by a descending method in a dark moist chamber for about 12 hours. The elutes, after adjusting its volume, were mixed with an equal volume of M/10 McIlvaine's citric acid-phosphate buffer, *p*H 5.0. The Hitachi fluorimeter (light source: mercury vapor lamp SHL-100 UV; primary filter UV-D2; secondary filter Hitachi #46 (460 mμ)) was used. The fluorescent intensity of 1.0 μg per ml of quinine sulfate standard in 0.1N H_2SO_4 was equivalent to that of 0.508 μg per ml of 7-hydroxybiopterin and 0.551 μg per ml of isoxanthopterin in McIlvaine's buffer solution, *p*H 5.0, respectively. As Cyprino-pourpre C has not yet been clarified in its chemical structure, the fluorescent intensity was represented by that of quinine μg in the above mentioned condition. The recovery rate was 72.2 percent for 7-hydroxybiopterin and 73.5 percent for isoxanthopterin in a system of the liver of goldfish and 7-hydroxybiopterin or synthetic isoxanthopterin of suitable concentration.

RESULTS

Pterin fraction in the larvae and in the adult skin of normal black (tt) *and transparent* (TT) *scaled types*

The detection and identification of fluorescent substances were carried out mainly by paper chromatography. Developing solvents were as follows: *n*-propanol-1% NH_4OH (2:1), *n*-butanol-alcohol-water (4:1:1), *n*-butanol-acetic acid-water (4:1:1), *n*-propanol-pyridine-water (5:3:2), three percent sodium citrate, five percent acetic acid, three percent NH_4Cl, five percent Na_2HPO_4 and three percent urea. It seems advisable to explain the results by division of them into the following four stages with melanophores being adopted as a marker for convenience: (1) melanophore-free stage, (2) early melanophore stage, (3) late melanophore stage and (4) adult form and adult (Tables 3 and 4 and Figure 2).

(1) Melanophore-free stage: The eggs and embryos from early morula to the stage immediately before the pigmentation at eye cup were examined. Two yellow fluorescent substances were detected and were identified as FMN and FAD, respectively, in an excellent developing solvent, 2,4-lutidine-*n*-butanol-water (4:1:1) (YAMAGIRI 1957), or by a hydrolytic treatment of them. No qualitative or quantitative differences in these substances were found in the two types. No pterins were found.

(2) Early melanophore stage: This stage covers the period from the first melanophore pigmentation at eye cup to the hatching stage. As described before, no difference was found with reference to the pigmentation in the two types. Paper chromatographic assay confirms the same sorts and quantitative similarity of pterins from the size and intensity of fluorescing spots in both types. Simultaneously, with the first appearance of pigmentation, blue, yellow and violet fluorescent pterins appeared successively. These three corresponded to biopterin (Rana-chrome 1), Rhacophoro-jaune and 7-hydroxybiopterin (Cyprino-pourpre

TABLE 3

Developmental stages and experimental conditions with reference to the normal black (tt) *and transparent* (TT) *scaled types*

Developmental stages (Age at about 21°C)	Whole body length (mm)	Number of individuals used for paper chromatography*
(1) Melanophore-free stage		
Morula (2.5 hours after spawning)	0.6	500
Gastrula (8 hours after spawning)	0.6	500
Trunk formation (20 hours after spawning)	0.6	500
(2) Early melanophore stage		
Eye cup becomes pigmented. (36 hours after spawning)	0.6	500
Hatching (3.5–4.0 days after spawning)	4.0–4.2	500*
(3) Late melanophore stage		
Five days after hatching (Both types cannot be distinguished.)	5.0–5.5	250
15 days after hatching (Both types can be distinguished through the pigmentation.)	8.0–9.0	250*
40 days after hatching (Depigmentation is not complete in the *TT* type.)	14–15	20*
(4) Adult form and adult		
40 days after hatching (Depigmentation is complete in the *TT* type.)	14–15	20*
Two years old	55–60	3

* Also for quantitative estimation.

A2), respectively. Subsequently, Cyprino-pourpre C and, a little later, isoxanthopterin (Cyprino-pourpre A1) appeared. The appearance of the last corresponded approximately to the period where guanophores appeared. Here, it is noted from the phylogenic standpoint that the sequence in which these pterins successively appear is definite notwithstanding the difference of species, e.g., the red scaled type of goldfish, carp or *Tribolodon hakuensis* (a kind of rudd, Cyprinidae).

(3) Late melanophore stage: This stage covers the period from the two-day stage to the 40-day stage after hatching. In the 40-day stage, the larvae are transforming into the adult chromatophore coloration, the *tt* type being heavily pigmented in black and the *TT* type slightly pigmented or already completely depigmented. In this section, only pigmented larvae were selected. Four violet, three blue, two yellow and one red fluorescing spots were detected on the chromatogram. The four violet corresponded to isoxanthopterin, 7-hydroxybiopterin, isoxanthopterin-6-carboxylic acid and Cyprino-pourpre C, the three blue to biopterin, Ranachrome 3, and 2-amino-4-hydroxy-6-carboxypteridine, the two yellow to ribo-

TABLE 4

Rf values of fluorescent substances in the normal black (tt) *and transparent* (TT) *scaled types (developed at 17°C, in the dark, ascending method)*

Spot number in Figure 2	Rf values in various solvents* a	b	c	d	e	Identification
1	0.38	0.34	0.40	0.78	0.75	Cyprino-pourpre C
7	0.20	0.14	0.18	0.60	0.56	7-Hydroxybiopterin
9	0.13	0.14	0.16	0.30	0.32	Isoxanthopterin
13	0.02	0.01	0.06	0.38	0.59	Isoxanthopterin-6-carboxylic acid
3	0.34	0.36	0.30	0.67	0.65	Biopterin
5	0.26	0.27	0.24	0.52	0.52	Rana-chrome 3
10	0.10	0.03	0.12	0.46	0.49	2-Amino-4-hydroxy-6-carboxypteridine
2	0.36	0.51	0.31	0.32	0.32	Rhacophoro-jaune
4	0.31	0.57	0.19	0.33	0.32	Riboflavin
6	0.24	0.01	0.04	0.57	0.54	FMN
12	0.09	0.09	0.01	0.45	0.38	FAD
8	0.14	0.07	0.29	0.42	0.42	Xanthopterin
11	0.09	...	0.09	...	...	Cyprino-rouge

* Solvents: (a) *n*-Propanol-1% ammonia (2:1); (b) 2,4-Lutidine-*n*-butanol-water (4:1:1); (c) *n*-Butanol-acetic acid-water (4:1:1); (d) three percent NH_4Cl; (e) five percent sodium citrate.

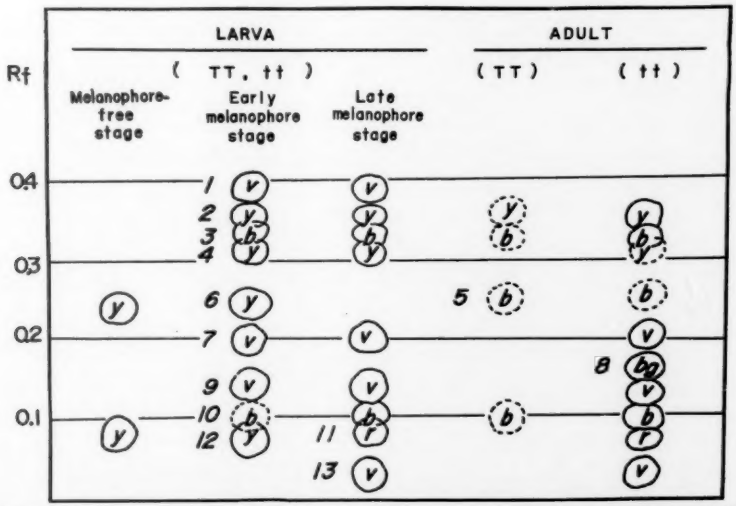

FIGURE 2.—Chromatogram showing the kinds of pterins in larva and adult of normal black *(tt)* and transparent *(TT)* scaled types. Solvent: *n*-Propanol-1% NH_4OH (2:1), at 17°C in the dark, ascending method. Color of fluorescence: b = blue; bg = bluish green; r = red; v = violet; y = yellow. Broken circle: faint fluorescence.

flavin and Rhacophoro-jaune, the one red to Cyprino-rouge, respectively. Whether the last is a pterin remains unsettled. The kind of spots was similar in both types, but the amount of pterins was in much less degree in the *TT* type, the fluorescing intensity of respective spots being proportional to the degree of pigmentation. During depigmentation, violet fluorescent substances disappeared in the following sequence: namely, Cyprino-pourpre C, a little later isoxanthopterin, and subsequently 7-hydroxybiopterin.

(4) Adult form and adult: The adult form denotes the *TT* type completely depigmented 40 days after hatching. The chromatogram in adult form differed qualitatively in its figure in both types. 7-Hydroxybiopterin, isoxanthopterin and isoxanthopterin-6-carboxylic acid were the pterins which could be found in the *tt* type as well as in the red scaled type—Cyprino-pourpre C being absent in their adults. So Cyprino-pourpre C is a substance which is found only in their larval life. The *TT* type is characterized by the complete absence of these pterins. However, if a large amount of skin is used, three blue, two yellow and one bluish green fluorescent substances can be unfailingly detected in the *tt* type fish. The three blue correspond to biopterin, Rana-chrome 3 and 2-amino-4-hydroxy-6-carboxypteridine, the two yellow to riboflavin and Rhacophoro-jaune, and the one bluish green to xanthopterin. Xanthopterin is scarcely found in the adult skin of the red scaled type fish. Only a trace of these substances is present in the *TT* type fish.

Quantitative estimation of isoxanthopterin, 7-hydroxybiopterin and Cyprino-pourpre C in the normal black and transparent scaled types

The estimation was carried out in the larvae at hatching, at 15-day and at 40-day stages after hatching. The contents and their mutual relation of 7-hydroxybiopterin, isoxanthopterin and Cyprino-pourpre C in a single fish in both types are given in Table 5 and Figure 3. As described before, Cyprino-pourpre C alone cannot be represented with a molar concentration. As the table and figure indicate, the quantity of pterins is proportional to the degree of pigmentation. At hatching stage, no difference was found in the two types. This datum is in accordance with the morphological observation of similar pigmentation in the two types. At the 15-day stage, a clear difference between the two types was found. In fact, the larvae of both types can be clearly distinguished by their characteristic pigmentation. At the 40-day stage, the *tt* type fishes are heavily pigmented and the *TT* type are perfectly depigmented or in the process of depigmentation. The estimation in the latter type, then, was done by dividing them into two kinds on the basis of being completely or incompletely depigmented. Remarkable differences were found in the two types. The quantitative difference in pterins in the *TT* type corresponded to that of pigmentation of the two groups. From the above description, it can be concluded that there exists an apparent correlation between the degree of pigmentation and the quantity of pterins.

TABLE 5

Quantitative estimation of violet fluorescent pterins in the postembryonic larvae of the normal black (tt) *and transparent* (TT) *scaled types*

Type	Number of individuals (wet weight mg)	Number of experiments	Contents per individual: 7-Hydroxybiopterin 10^{-3} μg quinine	7-Hydroxybiopterin 10^{-6} moles*	Isoxanthopterin 10^{-3} μg quinine	Isoxanthopterin 10^{-6} moles*	Cyprino-pourpre C 10^{-3} μg quinine
			Hatching stage				
tt	500 (561±2)	2	95±3†	0.19	15±1	0.05	11±2
TT	500 (568±3)	2	99±4	0.20	12±3	0.04	9±1
			15-day stage after hatching				
tt	250 (535±7)	2	824±8	1.66	94±4	0.29	72±4
TT	250 (568±8)	2	432±13	0.87	45±11	0.14	29±2
			40-day stage after hatching				
tt	20 (386±10) (heavily pigmented)	3	3107±96	6.25	386±57	1.19	574±23
TT	20 (383±40) (incompletely depigmented)	3	192±19	0.38	57±11	0.18	38±5
TT	20 (519±28) (completely depigmented)	2	103±12	0.21	0	0	0

* Calculation formula:
Molar concentration of 7-hydroxybiopterin or (isoxanthopterin) per individual. = μg quinine of 7-hydroxybiopterin or (isoxanthopterin) per individual. × $2.01 \cdot 10^{-6}$ or $(3.07 \cdot 10^{-6})$

The standard pterin solutions used for this calculation were prepared by the spectrophotometric method using TSCHESCHE's data on synthetic 7-hydroxybiopterin as a basis for Cyprino-pourpre A2 ($\log \varepsilon = 4.16$ in 0.05N NaOH at 342 mμ, mol wt 252.2) and employing synthetic isoxanthopterin (mol wt 179.1) for Cyprino-pourpre A1.

† mean ± standard deviation.

DISCUSSION

Among the various kinds of chromatophores, guanophores are almost devoid of pterins in the ventral white skin of the frog as well as in goldfish. It will be true to say that the presence of 7-hydroxybiopterin, isoxanthopterin and isoxanthopterin-6-carboxylic acid in the adult skin of red scaled goldfish is due to the xanthophores (allophores). Whether these substances, or some of these, exist also in the melanophores has not yet been demonstrated. At any rate, the above considerations are concerned with the adult chromatophores, and not the larval ones. The presence of the larval melanophores, which depigment during development (as clearly seen in the red scaled type fish), has been recognized by many workers (FUKUI 1927; GOODRICH and HANSEN 1931). On the contrary, the behavior attendant upon development of xanthophores has been scarcely reported. It has been reported that the difference between the erythrophores, characteristic of adult, and the xanthophores depends solely on the color of pigments within the

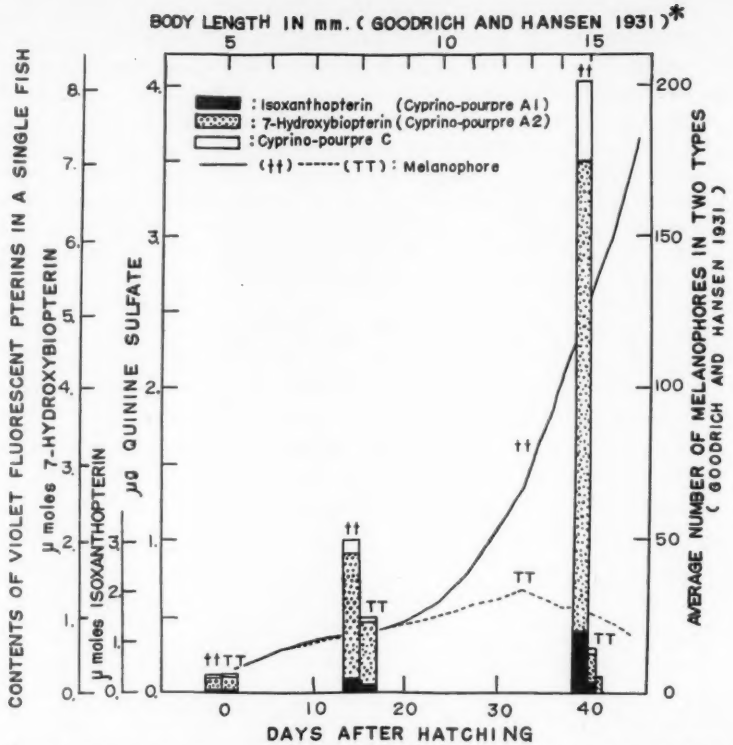

FIGURE 3.—Graph of contents of violet fluorescent pterins in a single fish of normal black *(tt)* and transparent *(TT)* scaled types.

* Slightly modified.

cell (PARKER 1948). According to our experience, red or orange pigments are present in every xanthophore of the adult, but not in those of larva. On the basis of this difference and owing to the fate of xanthophores in the larval stage of transparent scaled type fish, it is rather reasonable to discriminate between the two kinds of larval and adult types in xanthophores as well as in melanophores.

As described before, the numbers of melanophores, guanophores and xanthophores in the transparent type increase at a slow rate up to 10–13 days after hatching, followed by depigmentation (some difference in days is found). Thus, the fish becomes quite transparent. GOODRICH and HANSEN (1931) have previously clarified the difference of depigmentation between the red scaled and transparent scaled types by determinating the change in number of larval melanophores in the course of development. The graph presented by them is presented in modified form in Figure 3. This figure is concerned with melanophores, but the same holds approximately true for the xanthophores from our experience.

It is clear from the data of GOODRICH and HANSEN (1931) that the pterin contents are nearly proportional to the number of melanophores. Whether the pterins are related to either melanophores or xanthophores or to both has not been demonstrated. To obtain a decided conclusion, the localization of pterins in the chromatophores in question should be cleared up. From the above descriptions, it is suggested that gene action has its effect on the simultaneous disappearance of pigmentation and pterins in the chromatophores in the *TT* type fish. In fact, at the depigmentation of guanophores in this type, a conspicuous guanase activity was demonstrated by one of the authors (KAJISHIMA 1960).

SUMMARY

A relation between pigmentation and pterin contents in chromatophores was investigated employing the normal black scaled (*tt*) and transparent scaled (*TT*) types of goldfish which differ genetically in the mode of pigmentation during development.

1. The process of appearance and depigmentation of melanophores, guanophores and xanthophores in the course of development was described.

2. The pterin fractions in the course of development was examined by paper chromatography. Simultaneously, with the first pigmentation in the embryos, pterins such as biopterin (Rana-chrome 1), Rhacophoro-jaune, 7-hydroxybiopterin (Cyprino-pourpre A2, Ichthyopterin), Cyprino-pourpe C, isoxanthopterin (Cyprino-pourpre A1) and isoxanthopterin-6-carboxylic acid (Cyprino-pourpre B) appear in the same way in the two types. Whereas the violet fluorescent pterins of 7-hydroxybiopterin, isoxanthopterin and isoxanthopterin-6-carboxylic acid continue to remain in the adult skin of the *tt* type, no pterins are found in the transparent *TT* type. Cyprino-pourpre C is found only in the larva.

3. The fluorimetric estimation of the three main components in the larval life, 7-hydroxybiopterin, isoxanthopterin and Cyprino-pourpre C, was compared at respective stages of development in both types. No difference was detected at hatching stage where the embryos assume morphologically the same pigmentation. A clear difference was found at the 15-day stage where the embryos can be distinguished through pigmentation. Large amounts of pterin were found in the *tt* type fish (heavily pigmented), while little or no pterins were found in the *TT* type fishes which were depigmented incompletely or completely. These data indicate a close relation between the degree of pigmentation and the pterin contents of the chromatophores.

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CYTOGENETIC ANALYSIS OF THE ACTION OF CARCINOGENS AND TUMOR INHIBITORS IN DROSOPHILA MELANOGASTER.

VIII. SELECTIVE MUTAGENIC ACTIVITY OF S-2-CHLOROETHYLCYSTEINE ON THE SPERMATOGONIAL STAGES

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THE greatest majority of the physical and chemical mutagens so far investigated in Drosophila proved to be far more active on the postmeiotic stages of spermatogenesis than on the premeiotic cells (X-radiation: LÜNING 1952; various alkylating compounds: BIRD and FAHMY 1953; FAHMY and FAHMY 1954, 1955, 1957a, 1958a,b, 1960a). The mustard derivatives of naturally occurring amino acids deviated from this predominant pattern in so far as they proved to be mutagenetically active on all stages of the germ line, particularly the gonia (FAHMY and FAHMY 1960a). This selectivity for the anabolically active cells was attributed to "metabolic transport", whereby the amino acid mutagenic molecule could be conveyed through the cytosome to sites of nucleoprotein synthesis during chromosome replication. As a further test of this hypothesis, the mutagenicity of a monofunctional mustard derivative of a naturally occurring amino acid, *viz.* S-2-chloroethylcysteine, was analyzed. It was thought possible that in a molecule with a single alkylating group (the chloroethyl radical), the relative rôle of the alkylating to the prosthetic moiety in mutagenesis may be weighted in favor of the latter, thus exaggerating selectivity of action on the metabolically active cells.

The mutagenic cell-stage response of S-2-chloroethylcysteine is also of interest in relation to the mode of action of 2-chloroethyl methanesulphonate (FAHMY and FAHMY 1956a). The activity of this sulphonate on spermatogonia, in contrast to several other mesyloxy-esters, suggested the production *in vivo* of a secondary mutagen, probably an amino acid mustard (FAHMY and FAHMY 1957a). Biochemical evidence (ROBERTS and WARWICK 1957, 1958) indicated that the secondary mutagen is likely to be S-2-chloroethylcysteine. The mutagenic properties of this compound were accordingly investigated, with a view to the further elucidation of the suggested *in vivo* conversion of 2-chloroethyl methanesulphonate.

MATERIAL AND TECHNIQUE

The mutagen used in this investigation is S-2-chloroethylcysteine, CB.1592: $ClCH_2CH_2SCH_2CH(NH_2)COOH$. The synthesis of this compound, as well as preliminary reports on its mutagenic activity have already been published (CON-

NORS and Ross 1958; FAHMY and FAHMY 1958c). For the mutagenicity tests, the compound was administered in solution as the hydrochloride, around and within the testes of adult Oregon-K flies by our microinjection technique (details in FAHMY and FAHMY 1960c).

The sex-linked recessive lethals and visibles were determined by the Muller-5 technique, and the method of scoring has already been detailed (FAHMY and FAHMY 1955). In these experiments the treated males were bred *en masse* with an equal number of females. In some experiments a simultaneous determination was undertaken of the mutation rates induced in the X and II chromosomes, which were recovered from the same population of treated cells. For this purpose the treated males were mated individually to females homozygous for a modified Muller-5 X chromosome */sc^{81} In-S w^{a}sc^8/* and with the second chromosome pair heterozygous for *Cy* (associated with inversion) and *BlL*. The F_1 female progeny was utilized in the assessment of the sex-linked recessive lethal rate by the usual Muller-5 procedure. The F_1 male sibs were mated individually to *Cy/BlL* females, and their heterozygous F_2 *Cy* progeny were then inbred *en masse*. The F_3 cultures were observed, and the complete absence of the wild type class was taken as an indication of the induction of a II chromosome recessive lethal. Separate records for each treated male were kept to enable the exclusion of individuals heterozygous for spontaneous lethals before treatment.

The progeny of the treated males was fractionated into broods according to our standard technique (FAHMY and FAHMY 1954, 1955). The age of the male at the time of treatment was fixed at 30 ± 5 hours after eclosion. The treated males were mated to a succession of virgin females at set periods of three days, and the mutation rates in the various broods were determined separately.

Some of the sex-linked lethals were placed genetically and analyzed cytologically for chromosome aberrations in the salivary chromosomes. The salivary glands of larvae heterozygous for the lethals were used for the cytological study. The chromosomes were prepared by the aceto-orcein squash technique.

OBSERVATIONS

The brood-mutation curve: The mutation rates in the successive broods of the fractionated progeny of the treated males are presented in Tables 1 and 2 and Figure 1. The shape of the brood-mutation curve is characteristic and is fundamentally the same for the X chromosome and the second autosome. The mutation rate remains fairly constant and at a low level for three or four broods, and then rises sharply for the later progeny. This demonstrates that the stages of spermatogenesis can be broadly classified into two categories as regards their response to S-chloroethylcysteine: low sensitivity stages which are used during the first few days after treatment, and highly sensitive stages which are utilized in the later broods.

The point of upward flexion in the brood-mutation curves (Figure 1), due to the transition in the utilization of stages of markedly different sensitivity, varied with the method of breeding. In mass-mating experiments the transition point

TABLE 1

Sex-linked recessive lethals and visibles in successive broods at different doses

Conc. (× 10^{-2} M)	Brood (three days)	Mass matings Chr. tested	Mass matings Lethals No.	Mass matings Lethals Percent	Mass matings Visibles No.	Mass matings Visibles Percent	Pair matings Chr. tested	Pair matings Lethals No.	Pair matings Lethals Percent	Pair matings Visibles No.	Pair matings Visibles Percent
0.19	I	532	...	...	..	..	483	..	...	..	..
	II	531	8	1.5	2	0.4	503	1	0.2	..	..
	III	521	7	1.3	..	..	514	14	2.7	..	..
	IV	441	51	11.6	5	1.1	509	9	1.8	2	0.4
	V	499	119	23.8	25	5.0	472	51	10.8	23	4.9
	VI	503	24	4.8	26	5.2	495	41	8.3	20	4.0
	VII	61	8	13.1	1	1.6	299	34	11.4	16	5.4
	VIII	16	...	...	..	..	173	8	4.6	3	1.7
	Total	3104	217	7.0	59	1.9	3448	158	4.6	64	1.9
0.27	I	690	4	0.6	..	..	488	..	...	..	..
	II	664	7	1.1	..	..	427	4	0.9	1	0.2
	III	699	3	0.4	..	..	443	4	0.9	1	0.2
	IV	123	11	8.9	2	1.6	116	5	4.3	..	..
	V	201	30	14.9	4	2.0	403	29	7.2	15	3.7
	VI	60	9	15.0	..	..	286	57	19.9	10	3.5
	VII	638	75	11.8	35	5.5	...	..	...	..	..
	Total	3075	139	4.5	41	1.3	2163	99	4.6	27	1.3
0.41	I	498	3	0.6	1	0.2					
	II	498	8	1.6	2	0.4					
	III	446	3	0.7	..	..					
	IV	299	19	6.4	4	1.3					
	V	299	53	17.7	5	1.7					
	VI	147	44	29.9	16	10.9					
	VII	4	2	...	..	..					
	Total	2191	132	6.0	28	1.3					

TABLE 2

Second autosome recessive lethals in successive broods at different doses

Brood (three days)	Conc. 0.19 × 10^{-2} M Chr. tested	Lethals No.	Lethals Percent	Conc. 0.27 × 10^{-2} M Chr. tested	Lethals No.	Lethals Percent
I	271	1	0.4	143	..	...
II	297	2	0.7	369	3	0.8
III	260	12	4.6	445	14	3.1
IV	274	10	3.6	71	..	...
V	318	92	28.9	230	74	32.2
VI	346	139	40.1	156	75	48.1
VII	201	94	46.8	...	..	...
VIII	109	63	57.8	...	..	...
Total	2076	413	19.9	1414	166	11.7

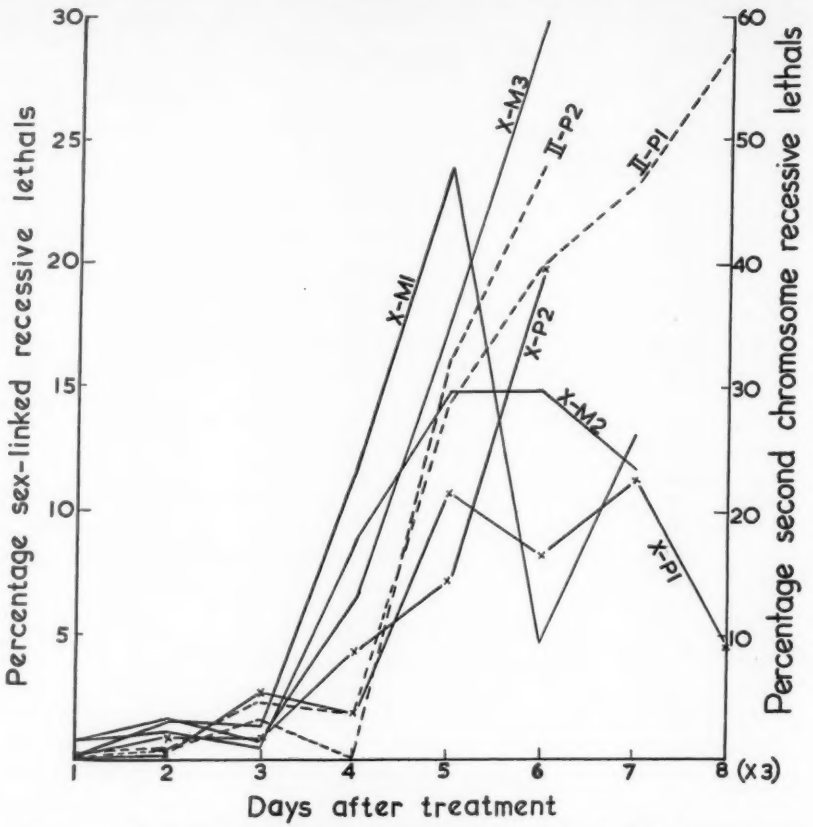

FIGURE 1.—The brood-mutation curves for the sex-linked (left ordinate) and second autosomal (right ordinate) recessive lethals. X chromosome experiments: continuous lines for mass matings; x——x for pair matings. Second chromosome experiments in dashed lines.

invariably occurred on the ninth day after treatment, indicating that the sensitive stages start to be recovered in the fourth brood. In pair matings, on the other hand, the transition occurred on the 12th day in three experiments, showing that the utilization of the sensitive cells had been delayed till the fifth brood. In the fourth experiment, however, (curve X-P2, Figure 1) the rise in mutation rate actually occurred in the fourth brood for the X chromosome mutations, but not for the concomitant second chromosome test. While the reason for this is unknown, it nevertheless shows that the effect of the breeding procedure (whether in pairs or *en masse*) is perhaps not crucial. The over-all picture from all experiments is that the cells sensitive to the cysteine mustard start to be utilized in the fourth or fifth

broods (10–15 days after treatment), with a higher tendency towards the end of this period (13–15 days).

The statistical analysis of the fluctuations in the mutation rates in successive broods was undertaken by YATES (1955) χ-method for the combination of probabilities from a series of 2×2 tables. The low mutation rates in the first three broods necessitated the pooling of data from this early progeny. The analysis (Table 3) indicates clearly the high significance of the brood-mutation pattern detailed above. One major difference, however, occurs between the X and second chromosome experiments. The mutation rate of the autosomes shows a consistent increase in the later broods (from the fifth onwards), whereas that of the sex-chromosomes reveals marked fluctuations.

Brood cell-stage relationship: The interpretation of the brood-mutation curve in terms of cell-stage sensitivity necessitates an exact determination of the time of sampling of the various stages of the germ line. It is, of course, clear that the least sensitive stages are those utilized in the earlier broods, which must have been sperm or maturing spermatids at the time of treatment. Equally certain is the fact that the most sensitive stages gave the sperm for the latest progeny and must have been mainly spermatogonial stages. There remains to be determined the exact moment in spermatogenesis when the transition occurs from low to high sensitivity. This was approached through an examination of the correlation between the time of the rise in the mutation rate and the start of the utilization of sperm derived from proliferating spermatogonia.

A mutation induced in a spermatogonium will be repeatedly multiplied through the successive mitoses and will ultimately lead to the production of a bunch of sperm carrying the same mutant. It would follow, therefore, that the first appearance of mutant "clusters" in the fractionated progeny of the treated males will

TABLE 3

Distribution of χ for the increase in mutation rate in consecutive broods

Exp.	Conc. ($\times 10^{-2}$ M)	Broods				
		1st III–IV	IV–V	V–VI	VI–VII	VII–VIII
Mass matings						
X–M1	0.19	11.1	4.9	−8.6	2.7	..
M2	0.27	8.4	1.6	0.0	−0.7	..
M3	0.41	6.2	4.3	2.9	0.9	..
Total		25.7	10.8	−5.7	2.9	..
P		$<10^{-9}$	$\simeq 10^{-9}$	<0.001	0.05	..
Pair matings						
X–P1	0.19	1.4	5.9	−1.3	1.4	−2.5
P2	0.27	4.1	1.1	5.0	..	..
Total		5.5	7.0	3.7	1.4	−2.5
P		$<10^{-4}$	$<10^{-6}$	0.004	0.08	0.006
II–P1	0.19	1.8	8.1	3.0	1.5	1.9
P2	0.27	..	..	3.1	..	..
Total		1.8	8.1	6.1	1.5	1.9
P		0.04	$<10^{-9}$	$\simeq 10^{-9}$	0.07	0.03

be a marker for the utilization of sperm derived from treated spermatogonia still capable of mitoses. Mutant clusters were scored among the sex-linked recessive visibles, and were also tested for among second chromosome lethals from a sample of the treated males (Table 4). No mutant clusters occurred during the first four broods, but they started to appear in the fifth brood and tended to increase in frequency in the later progeny. The size of the individual clusters also became larger in the sixth and later broods. The largest bunch recovered comprised 19 mutants and extended over three broods (sixth–eighth inclusive).

It can safely be concluded, therefore, that sperm derived from treated spermatogonia (before the growth period, while the cells are capable of multiplication) start to be utilized appreciably from the fifth brood, 13–15 days after treatment. This means that in the preceeding four broods the postspermatogonial stages of the germ line are used. It should be noted, however, that the absence of clusters among the small sample of mutants recovered from the fourth brood is not conclusive evidence against the utilization of some proliferating spermatogonia. This absence does not reach statistical significance as compared to cluster recovery in the fifth brood ($P = 0.28$). Furthermore, spermatogonia which mutated towards the end of their mitotic proliferation could only result in the production of a few sperm with the same mutation, which will naturally lower the probability of mutant recovery in clusters.

The above considerations indicate that in the present fractionation experiments the sperm and the various stages of spermateleosis are mainly utilized during the first three broods, 0–9 days after treatment. In the fourth brood (10–12 days) the situation is not very clear, but it is highly probable that the sperm for this period is derived from a mixture of cells: spermatocytes at various stages of meiosis and old spermatogonia towards the end of their proliferation cycle or immediately afterwards (auxocytes). For the fifth and later broods the situation is very clear and decisive. The sperm used in this late progeny is predominantly, if not exclusively, derived from young spermatogonia still capable of mitotic proliferation after treatment.

In the pair mating experiments, a substantial rise in mutation rate did occur

TABLE 4

The distribution of the single (s) and "cluster" (c) mutants in the successive broods. The number of mutants involved in the clusters are bracketed

Experiment	Mutants tested	Broods I–IV s	I–IV c	V s	V c	VI s	VI c	VII s	VII c	VIII s	VIII c	Overlapping clusters V +
X–M1	59	7	..	11	4(10)	2	6(21)	1	....	.	....	3(7)
M2	41	2	..	3	....	.	....	1	7(31)	.	....	1(4)
M3	28	7	..	1	....	3	2(9)	.	....	.	....	2(8)
X–P1	64	2	..	13	1(8)	7	4(11)	5	2(5)	1	....	2(12)
P2	27	2	..	8	2(6)	5	1(4)	.	....		....	1(2)
II–P1	143	3	..	7	1(3)	9	4(13)	6	1(2)	10	6(24)	8(66)
Total	362	23	..	43	8(27)	26	17(58)	13	10(38)	11	6(24)	17(99)

in the fifth brood, coincident with the first utilization of sperm from treated young spermatogonia. This shows that S-chloroethylcysteine is almost exclusively active on proliferating spermatogonia. On the other hand, in mass mating experiments, the rise in mutation rate occurred in the fourth brood. This could best be explained as due to a speedier differentiation of the germ line (perhaps as a result of a higher mating frequency in mass cultures) leading to an earlier recovery of some of the responsive young spermatogonia.

Relative mutability in the X and second chromosomes: In the X chromosome experiments, the mutation rate tended to remain the same, or even to fall slightly, after the fifth brood. In contrast, the second chromosome mutation rate showed a continuous rise from the fifth brood throughout the later progeny. This immediately suggested germinal selection against the hemizygous mutations induced in the early germ cells. An attempt was then made to analyze the exact time of onset and the degree of germinal selection in the various progeny fractions. For this purpose a study was undertaken of the relative mutation rates in the successive progeny fractions for the X and second chromosomes from the same treated cell population. Chromosome II is double the length of the X and would, therefore, be expected to yield twice its mutation frequency for the same dose. Table 5 gives the χ distribution for the deviation of the observed mutation frequencies for the two chromosomes from the theoretical 2:1 ratio for the successive broods. There was no significant deviation from expectation for the first four broods. In the fifth and later broods, however, a very gross excess of second chromosome mutants were recovered. Germinal selection, therefore, starts to operate against the recovery of some of the hemizygous lethals induced in the germ cells providing the fifth brood, which, as deduced earlier, includes proliferating spermatogonia. The more pronounced shortage of sex-linked lethals in the sixth and later broods indicates that the effect of germinal selection becomes more drastic the younger are the mutated gonia and the longer is the period needed for their differentiation.

Visible to lethal ratio: Visible mutations were only scored in the X chromosome experiments (Table 1). It is quite obvious that their yield in the successive broods follows roughly the same pattern as that of the lethals. The visible rate is extremely low for the first four broods and then becomes appreciably higher in the later progeny. It is virtually certain, therefore, that the visibles, like the lethals, are

TABLE 5

Distribution of χ for the comparison of the mutation rates in the second:X ($\times 2$) chromosomes for different broods from concomitant experiments

Conc. ($\times 10^{-3}$ M)	Broods					
	I–III	IV	V	VI	VII	VIII
0.19	−0.3	0.1	2.3	7.7	5.7	8.8
0.27	1.2	..	5.3	2.4	..	..
$\Sigma\chi$	0.9	0.1	7.6	10.1	5.7	8.8
P	0.3	0.5	$<10^{-7}$	$<10^{-9}$	$<10^{-8}$	$<10^{-9}$

almost exclusively induced in the spermatogonial stages. This makes it almost impossible to determine the visible to lethal ratio characteristic of the cysteine mustard. The mutants from the insensitive postmeiotic stages are too few to give a reliable ratio, and those from the sensitive spermatogonia are bound to show a distorted value in favor of the visibles, because of germinal selection against the lethals. The analysis of the variation of the visible to lethal ratio in the successive broods, however, could be of some value in the understanding of the relative recovery of the two mutant types induced in spermatogonial stages. To make such an analysis statistically feasible, extensive pooling was necessary. Mutants recovered in the same brood in different experiments were summed, and the first three broods derived from the low sensitivity stages were grouped as a single fraction (Table 6). Such a method of pooling presumes that the ratio of visibles to lethals is independent of dose and postmeiotic cell stage. While it was not possible to confirm this property for the cysteine mustard, because of the complications of germinal selection, there is no reason to doubt its reality. Ample evidence is now available (FAHMY and FAHMY 1955, 1960b) that whenever germinal selection is not pronounced, the same compound induces a constant and characteristic visible/lethal ratio.

The statistical analysis of the fluctuation of the visible to lethal ratio in the various fractions of the germ line is given in Table 6. This ratio shows the first significant change in favor of visibles between the fourth and fifth broods. The proportion of visibles is even greater in the sixth brood, and the high ratio is maintained in later progeny. This is further evidence that germinal selection operates against the recovery of the hemizygous lethals from proliferating spermatogonia.

Effect of dose: The various doses investigated, and their range, do not permit an exact determination of the relationship between the concentration administered and the mutation rate recovered. There are sufficient data, however, to assess whether a dose effect occurs. This was tested statistically by the x method (Table 7). Evidence for a dose effect occurred in the comparison involving the X chromosome experiments with dose doubling (X-M1, and M3). There was also suggestive evidence for a dose effect in two of the comparisons where the dose

TABLE 6

Variation in the over-all sex-linked recessive visible to lethal ratio in different germ-cell fractions

Brood	Visibles	Lethals	Total	Ratio v/l	χ^2
I–III	7	66	73	0.11	
					0.3
IV	13	95	108	0.14	
					3.8
V	72	282	354	0.26	
					6.2
VI	72	175	247	0.41	
					0.1
VII	52	119	171	0.44	

ratio was 1.4 (X-P1 and P2; II-P1 and P2), but not in the third (X-M1 and M2). The most interesting fact that emerges from the analysis is that a very decisive and highly significant dose effect invariably occurs in the sixth brood, which is almost exclusively derived from treated proliferating spermatogonia.

The nature of the lethals: A random sample of 102 sex-linked recessive lethals induced by S-chloroethylcysteine was analyzed cytologically for detectable chromosome aberrations in the salivary gland chromosomes. To make the sample representative, the mutants were drawn from the various X chromosome experiments; they were accordingly induced by various doses and were recovered from different broods. Since the cysteine mustard is preferentially active on the proliferating spermatogonia, it was essential to guard against examining "mutant clusters" of common origin. For mutations arising in the fourth and later broods, therefore, only one lethal per treated male was used in the cytological study. This meant the selection of a few lethals from cell samples with grossly different mutation rates. Under such circumstances it would be virtually impossible to give an accurate assessment of the mutagenic dose in the sample analyzed. A rough estimate of the strength of the treatment, however, was calculated as the weighted mean mutation rate in the broods from which the observed mutants were drawn; the weight was taken as the number of mutants selected from the same brood.

Table 8 shows the summary of the data with the monofunctional cysteine mustard, as compared with those obtained with a difunctional mustard derivative of another amino acid, *viz.:* L-phenylalanine. The mutagenic dose for the two

TABLE 7

Distribution of χ for the effect of dose on mutation rate in the various broods of comparable fractionation experiments

Comparison	Broods I–III	IV	V	VI	$\Sigma\chi$	P
X–M1 and M2	−0.9	−0.8	−2.6	3.2	−1.4	0.2
X–M1 and M3	0.1	−2.4	−2.0	8.8	4.5	0.01
X–P1 and P2	−1.2	1.7	−1.8	4.7	3.4	0.04
II–P1 and P2	−0.1	..	0.8	1.7	2.4	0.08
$\Sigma\chi$	−2.1	−1.5	−5.6	18.4	..	..
P	0.1	0.2	0.003	$<10^{-9}$	..	..

TABLE 8

Chromosome aberrations among sex-linked recessive lethals induced by S-chloroethylcysteine (CB.1592) and p-N-di-(2-chloroethyl)aminophenylalanine (CB.3025)

Compound	Stocks examined	Lethal rate percent	Gross rearrangements No.	Gross rearrangements Percent	Deficiencies No.	Deficiencies Percent
CB.1592	102	6.0	1	0.9	23	22.5
CB.3025	134	7.0	3	2.2	50	37.3

compounds can be looked upon as roughly the same. It is abundantly clear that both agents are very weak chromosome breakers. The single major rearrangement observed in the cysteine mustard sample was a deletion, where 21 bands were eliminated. It was recovered from the third brood and was probably, therefore, induced in a spermatid. It is perhaps noteworthy that no major rearrangements were induced in the premeiotic stages, in spite of the fact that the lethal rate in some of these cell samples reached a maximum of 20 percent. It would seem highly probable, therefore, that the cysteine mustard is totally ineffective in breaking the chromosomes of spermatogonial stages.

Small deficiencies (involving less than one percent of the length of the salivary X chromosome) were induced by the cysteine mustard at an over-all rate of 22.5 percent which is significantly lower than that under the phenylalanine derivative ($\chi^2 = 5.9$ for one degree of freedom, $P \simeq 0.01$). This may indicate that monofunctional mutagenic agents are less effective in the induction of small deficiencies than are the difunctional compounds of the same chemical series.

DISCUSSION

S-2-chloroethylcysteine is the first instance so far discovered of a mutagen which is virtually specific to a particular cell stage of the male germ line. The compound is ineffective on mature sperm, produces a very low activity on the stages of spermateleosis, but is extremely effective on the early germ cells—particularly the earlier spermatogonia; the mutation rate in these early cells sometimes reaches twenty-fold that of the spermatids. Advantage was taken of the high mutagenic selectivity of the cysteine mustard on the spermatogonia, to determine the start of utilization of these cells in our standard brood-fractionation experiments (see technique). For this purpose three independent criteria indicative of the utilization of mutated spermatogonia were investigated: (1) the appearance of "clusters" of identical mutants, (2) the disturbance of the sex-linked visible/lethal ratio, (3) the recovery of the X, relative to the second chromosome lethals. The evidence from the above sources pointed to the same conclusion: the first appreciable utilization of proliferating spermatogonia occurs in the fifth brood (13–15 days after treatment), coincident with the marked rise in the mutation rate. There were indications, however, that some of the spermatogonia may also be used in the fourth brood in admixture with the youngest spermatocytes (auxocytes) in the premeiotic interphase. The pattern of cell-stage utilization in the present brood-fractionation experiments with the cysteine mustard, therefore, is essentially the same as that occurring in comparable experiments with other alkylating agents (FAHMY and FAHMY 1955, 1957a, 1960a). This adds further support to the conclusion (FAHMY and FAHMY 1958c) that, with moderate doses of the alkylating mutagens and under standard experimental conditions, the speed of germ line differentiation is not drastically disturbed, thus resulting in a reasonable consistency in the cell-stage/brood correlation in different fractionation experiments. Spermatogonial stages (capable of mitoses) are predominantly used starting from the fifth brood, whereas prespermatogonial stages are used in the earlier progeny.

The activity of the cysteine mustard on cells recovered in the late progeny might imply that it is a function of time, being due to the prolonged action of a slow reacting compound. This possibility can immediately be excluded on the basis of the known chemical properties of the compound. In buffered aqueous solution at *p*H 7.5 and 37°C the half life of chloroethylcysteine hydrochloride, with respect to the hydrolysis of the chloroethyl group, is seven minutes (W. DAVIS, in CONNORS and ROSS 1958). This means that in the present experiments the administered drug is probably completely inactivated by hydrolysis in the haemocoel of the flies in under the hour. The earliest sample of sperm tested in the genetic experiments was collected during the first three days after treatment, long after the complete inactivation of the compound. It would accordingly follow, that the observed fluctuations in the mutation rate in the various broods is a genuine manifestation of cell stage sensitivity. It is due to differential damage during the compound's period of activity and is independent of the sampling times in the genetic tests.

A surprising feature of the mode of action of S-chloroethylcysteine is its extremely low activity on the postmeiotic stages, particularly the mature sperm. Furthermore, the mutation rate in these stages did not manifest any dose response. These features could imply the lack of penetrance of the compound into the sperm and spermatids. This possibility is rendered most unlikely, however, by the fact that the compound does penetrate and mutate the spermatogonial stages whose nuclei are far better protected than those of the sperm by the surrounding cytoplasm. A more plausible explanation for the inactivity on the sperm is that the cysteine mustard may well be ineffective on the fully formed, highly condensed, chromosomes. Conversely, the compound would be expected to be most active on the attenuated chromosome. This would be in harmony with the agent's effectiveness on the genes of the proliferating spermatogonia. Maximal chromosome attenuation would naturally occur during the synthesis of the new replicate early in the mitotic cycle, and it is probable that this is the moment of the maximal sensitivity.

The recessive lethals induced by the cysteine mustard conform with those induced by other amino acid mustards (FAHMY and FAHMY 1956b, 1957b) in so far as they are virtually lacking in major chromosome aberrations. The cytological data with this mustard, however, while indicating that the agent is a very weak chromosome breaker, do not permit an accurate assessment of this property. A proportion of the rearrangement lethals, if induced in the premeiotic stages where the mutagenicity is highest, is bound to be eliminated by germinal selection. An interesting deviation of the cysteine-mustard lethals from those of other difunctional amino acids is that they are associated with a significantly lower rate of deficiencies and small deletions (not exceeding ten salivary chromosome bands). Among lethals induced at roughly the same over-all rate, there was nearly double the deficiency rate under the difunctional mustard derivative of phenylalanine as under the monofunctional cysteine derivative. Here, as in the case of major rearrangements, differential germinal selection could have depressed the rate of deficiency lethals recovered from spermatogonia, and the data

were not sufficient to determine its degree. Nevertheless, it is highly probable that the low deficiency rate under the monofunctional mustard is a genuine phenomenon and not merely an artifact due to selection. Recent work with the mono- and difunctional sulphonates which are both active on the postmeiotic stages (where no germinal selection occurs) yielded results parallel to the present data with the mustards. For the same mutagenic dose, the rate of deficiency lethals was far lower for the monofunctional than for the difunctional sulphonic esters (FAHMY and FAHMY, unpublished).

Reference has been given to the biochemical evidence that S-2-chloroethylcysteine could be one of the metabolites produced *in vivo* after the administration of 2-chloroethyl methanesulphonate. The present genetical evidence is in harmony with the biochemical findings. The mutagenic cell-stage response under the two compounds is strikingly comparable, particularly in so far as both are preferentially active on the proliferating spermatogonia. This similarity of pattern also supports the hypothesis that the deviation of the brood-mutation curve for the chloro-ester from the typical sulphonate pattern (with no activity on the gonia) could be due to the conversion of the sulphonate into the amino acid mustard *in vivo* (FAHMY and FAHMY 1957a, 1958c).

SUMMARY

The fractionated progeny of males injected with S-2-chloroethylcysteine showed a marked selective mutagenicity on the early germ cells, which start to be utilized 10–15 days after treatment. The sperm sample used towards the end of this period (13–15 days), (a) contained clusters of identical mutants, (b) showed a disturbed sex-linked visible to lethal ratio in favor of the visibles, and (c) gave a disproportionate excess of second to X chromosome lethals. This shows that the selective mutagenicity of the cysteine mustard is on the proliferating spermatogonia. The recessive lethals induced were virtually free of major rearrangements and were associated with a low rate of small deficiencies. The pattern of cell stage response of the cysteine mustard was similar to that of chloroethyl methanesulphonate.

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SEX BALANCE IN DROSOPHILA MELANOGASTER: ANEUPLOIDY OF LONG REGIONS OF CHROMOSOME 3, USING THE TRIPLOID METHOD[1]

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IN contrast with X chromosome triploid aneuploid studies (DOBZHANSKY and SCHULTZ 1934; PIPKIN 1940), alterations of dosage of any one short region of chromosome 2 (PIPKIN 1947) or of chromosome 3 (PIPKIN 1959) have revealed no pronounced sex shift in hyperintersexes or hypointersexes. Although the mean sex types of hyperintersexes bearing in excess of 2X3A a short right-hand end fragment of three different 3;4 translocations were slightly but significantly more male-like than the mean sex types of their control intersexes; hypointersexes for each of two components of this region showed no corresponding shift in the female direction (PIPKIN 1959). The purpose of the present investigation has been to study sex balance of long-region aneuploids in a further attempt to locate the autosomal chromosomal region responsible for the shift toward maleness in ordinary 2X3A triploid intersexes caused by the addition of an extra set of autosomes to the 2X2A female chromosome complement.

MATERIALS AND METHODS

Certain of the same 3;4 translocations used in the short-region aneuploid study (PIPKIN 1959) have been used in the present investigation of long regions. These include the following: T(3;4)c (DOBZHANSKY 1929a; LEWIS 1951), broken at 86C; T(3;4)A13, broken at 67E; T(3;4)A12, broken at 73C; T(3;4)A2, broken at 94A3-4; T(3;4)A28, broken at 94D3-4 (LEWIS, personal communication); T(3;4)A30, broken at 96E; all T(3;4)A translocations being studied by PATTERSON, STONE, BEDICHEK and SUCHE (1934) and PATTERSON, BROWN and STONE (1940); T(3;4)85C (LEWIS, personal communication), broken at 85C; T(3;4)89E (LEWIS, personal communication), broken at 89E; T(3;4)H1 and T(3;4)H3, each broken in the chromocenter; and T(3;4)H5, broken at 96E. The *y*^{2}*; ru ca* triploid stock used has been described previously (PIPKIN 1959). All of the experimental crosses were placed in a B.O.D. incubator to develop at 22 ± 0.5°C.

To study aneuploidy of long end regions, males heterozygous for a given 3;4 translocation carrying normal alleles and an intact third chromosome carrying the recessive markers *ru* (roughoid) and *ca* (claret) were crossed with homozygous *y*^{2}*; ru ca* triploid females according to Figure 1 (y^{2} = yellow). The possible surviving long-region aneuploid progeny, hypertriploid females and hyperinter-

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sexes, are shown in Figure 2. Hypertriploid females carrying a long left-end fragment appear simply *ca*. Hyperintersexes may be either *ca* or y^2; *ca*, depending on the parental source of the X chromosomes. Similarly, hypertriploid females carrying a long right-hand end fragment appear *ru;* such hyperintersexes, appear either *ru* or y^2; *ru*. To demonstrate that the surviving long fragment aneuploids are hypertriploids and hyperintersexes rather than hypotriploids and hypointersexes, a control cross was made as follows: Males with the given 3;4 translocation carrying normal alleles and an intact *Dcx* third chromosome (carrying the dominant marker *D*, Dichaete, associated with an inversion) were crossed with y^2; *ru ca* triploid females. Any triploid aneuploid progeny (distinguished by coarse wing texture and large eye facets) showing either *ru* or *ca* from this cross must be either hypotriploid females or hypointersexes since only two third chromosomes with recessive markers are derived from the y^2; *ru ca* triploid parents. Since no *ru* or *ca* aneuploid progeny appeared from any of these control crosses, the aneuploids appearing from the cross shown in Figure 1 must be hypertriploid females and hyperintersexes.

To study aneuploidy of interior long regions, use was made of the overlapping translocation method, first described by MULLER and STONE (1930), and employed in a number of aneuploid studies since. Homozygous y^2; *ru ca* triploid females were crossed with males heterozygous for two different 3;4 translocations. For long interior region studies, the translocation with breakage point to the left carried *ca* and the normal allele of *ru*, whereas the translocation with the breakage point to the right carried *ru* and the normal allele of *ca*. This procedure insured that possible hypertriploid females, which of all aneuploids are most apt to survive, would be *ru ca*. This method is diagrammed in Figure 3.

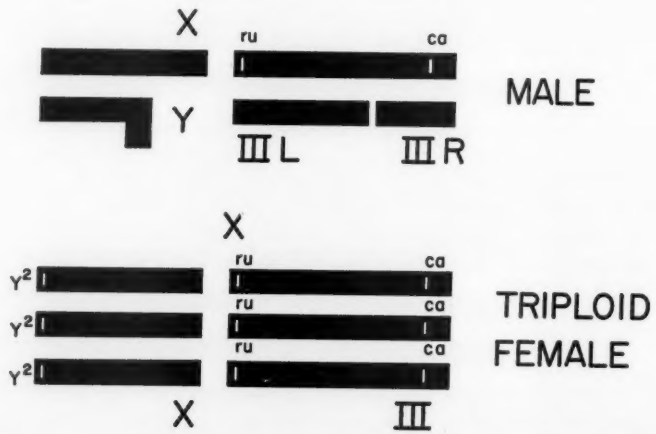

FIGURE 1.—Cross designed to produce hypertriploid and hyperintersex progeny carrying in excess of 3X3A or 2X3A, respectively, a long left-hand end fragment.

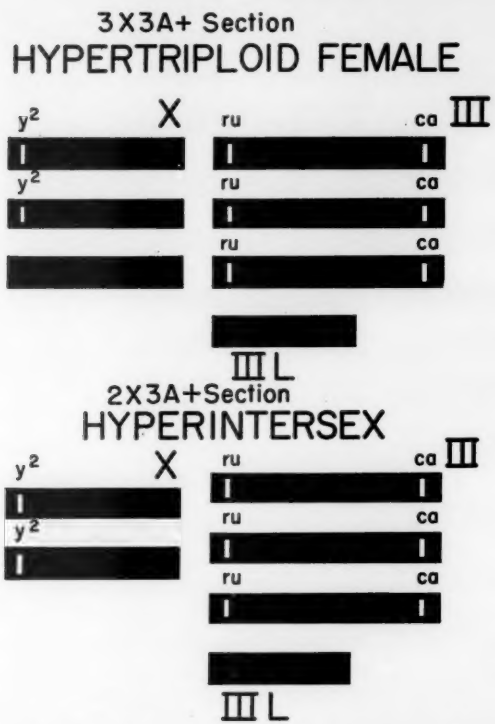

FIGURE 2.—Claret hypertriploid female and *y²; ca* hyperintersex, each carrying a long left-hand end fragment of chromosome 3.

EXPERIMENTAL RESULTS

Long end regions: A summary of long end region aneuploid studies is given in Table 1. The first column gives the genetic composition of the translocation-carrying male parent crossed with *y²; ru ca* triploid females. Numbers of aneuploid progeny and of wild-type control (2X3A) intersexes appear in Table 1. A comparison of the numbers of this one group of control intersexes with those of aneuploids indicates the rarity of the latter in the progeny of a particular experimental cross.

Table 1 shows that no hypertriploid female survived carrying either the left- or right-hand end fragment of either T(3;4)H1 or T(3;4)H3, each broken in the chromocenter. Hypertriploid females and hyperintersexes bearing left-hand end fragments of T(3;4)12, T(3;4)85C, and T(3;4)89E were found when rather high progeny counts were made of the experimental crosses indicated in the first column of Table 1. Aneuploid and control 2X3A sibling intersexes listed in Table

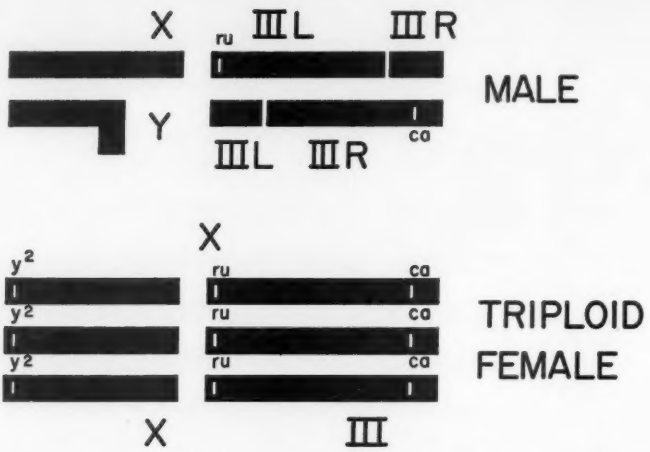

FIGURE 3.—Cross designed to produce hypertriploid or hyperintersex progeny carrying in excess of 3X3A or 2X3A, respectively, a long interior region of chromosome 3.

1 are classified according to the sex types I–V, described by DOBZHANSKY and SCHULTZ (1934). Owing to the low numbers of surviving long region hyperintersexes and to the fact that the control intersexes themselves were predominantly of the male types I and II, little information can be derived from a comparison of the sex types of the two groups. For this reason a detailed study was made of the sex combs of long- and short-region hyperintersexes, their respective control 2X3A sibling intersexes as well as of short region hyperdiploid males, control sibling males (X 2A), and supermales (X 3A) arising from crosses of males heterozygous for one or two 3;4 translocations and *y*^{2}*; ru ca* triploid females. The aneuploids, thus studied, occurred in the experimental crosses of the present investigation and also of the previous short-region aneuploid study of chromosome 3 (PIPKIN 1959). A detailed analysis of the sex comb data of these aneuploids will appear in a later paper. In no case did a long-region hyperintersex possess sex combs with a higher number of prongs (i.e., more male-like) than those of its corresponding control 2X3A sibling intersex.

Claret hypertriploid females carrying 12 L in excess of 3X3A are similar to their 3X3A sibling triploids in body size, eyes, wings, bristles, and sexual characters. Hyperintersexes with 12 L + 2X3A differ from their 2X3A control sibs by having thinner bristles, postverticals more often missing, and ocelli partly missing.

Claret hypertriploids carrying 85C L in addition to 3X3A were about the size of intersexes, had dark trident markings, wings sometimes imperfectly expanded, one posterior scutellar bristle occasionally missing, bristles thin. Wing texture was triploid in appearance, and eye facets were regular and triploid in size. No

TABLE 1

Aneuploidy involving long-end regions of chromosome 3

T(3,4) male parent × y^2; *ru ca* triploids	Hyperdiploid XY2A + L ♂ y^2; *ca*	Hyperdiploid 2X2A + L ♀ *ca*	Hyperdiploid 2X2A + L ♀ y^2; *ca*	Hyper-triploid ♀ 3X3A + L *ca*	Hyper-triploid ♀ 3X3A + R *ru*	Hyperintersex 2X3A + L *ca* or y^2; *ca*	Hyperintersex 2X3A + R *ru* or y^2; *ru*	Control intersex wild type I	II	III	IV	V
12/*ru ca*	1	18	8	19	0	11 I, 4 II (*ca*); 6 I, 2 II, 1 IV (y^2; *ca*)	0	302	106	40	54	2
H1/*ru ca*	0	0	0	0	0	0	1 I (*ru*)	212	69	22	42	2
H3/*ru ca*	0	0	0	0	0	0	0	184	63	25	65	2
85C/*ru ca*	0	0	0	23	1	9 I, 3 II, 1 III, 1 IV (*ca*); 2 I (y^2; *ca*)	2 IV (y^2; *ru*)	346	202	60	160	4
89E/*ru ca*	0	2X2A + R *ru* y^2; *ru* 21 2		12	24	1 III (*ca*)	1 III (*ru*); 1 I, 1 II, 1 IV (y^2; *ru*)	405	175	56	111	3

signs of intersexuality were present, but the hypertriploids were short-lived and none yielded progeny.

Hyperintersexes of the constitution 85C L + 2X3A were either yellow or wild type depending on the source of their X chromosomes. These hyperintersexes regularly lacked both pairs of scutellar bristles, had malformed ocelli, but otherwise were similar to 2X3A sib intersexes in body size, wing texture, and eyes. Dissection of an 85C L + 2X3A hyperintersex revealed a normal male duct system and small coiled testes.

The single 85C R + 3X3A hypertriploid female had wings held erect over the body, wing texture and eye facets triploid in appearance, and body size approximately that of an intersex. All sexual characters were female. Two hyperintersexes carrying the right-hand end of 85C in addition to 2X3A were found.

The 12 claret aneuploid females resulting from the cross of T(3;4)89E/ *ru ca* male with y^2; *ru ca* triploid females were characterized as follows: late hatching, each the size of an intersex, a shrunken abdomen, anterior ocellus missing, slender bristles, eye facets undisturbed but as small as in diploid females, wings deeply clipped medially, and wing texture as fine as that of diploid females. Sexual characters were female. Dissections revealed underdeveloped ovaries with no mature eggs. These 12 aneuploid females are presumed to be 89E + 3X3A hypertriploid females for the following reasons: First, no *ca* aneuploid females were found among the progeny of T(3;4)89E/ *ru ca* males with y^2; *ru ca* diploid females in a testcross designed to demonstrate whether or not hyperdiploid females of the genotype 89E L + 2X2A could survive. Hence, the 12 *ca* aneuploid females from the first cross (of T(3;4)89E/ *ru ca* males with y^2; *ru ca* triploid females) could not have been hyperdiploid females of genotype 89E L + 2X2A even though the wing texture and eye facets of the former were diploid in appearance. Second, since no *ca* aneuploid females resulted from a cross of T(3;4)89E/ *Dcx* males with y^2; *ru ca* triploid females, the *ca* aneuploid females of the first cross could not have been hypotriploid females. Finally, since the 12 *ca* aneuploid females of the first cross possessed eye facets with no irregularities in arrangement, they were not confused with "triple X" females of genotype 3X2A which also have medially clipped wings but, in addition, disturbed eye facets. Triple X (3X2A) females occurring in the progeny of the first cross were expected to be either wild type or *ru ca*. One such triple X (3X2A) *ru ca* female was found among the progeny of the first cross. With profoundly disturbed eye facets, this triple X female could not have been confused with its 12 sibling *ca* aneuploid females possessing undisturbed eye facets. The 12 *ca* aneuploid females are therefore thought to have been 89E + 3X3A hypertriploid females. Their small body size, fine wing texture, slow development, diploid facet size, and medially clipped wings must have been the result of genic imbalance.

Twenty-four *ru* hypertriploid females of the genotype 3X3A + 89E R were observed, as Table 1 shows. These late-hatching aneuploids possessed shrunken abdomens, invariably carried wings outstretched and held up over the body. The bristles were thin; eyes were typically roughoid but triploid in facet size. Wing

texture was coarse as in triploids. Sexual characters were female; ovaries, rudimentary.

Wings were widely outstretched in the *ru* hyperintersexes carrying 2X3A + 89E R. Otherwise these hyperintersexes resembled control sibling intersexes. Similarly, wings were widely outstretched in the single *ca* hyperintersex of the genotype 2X3A + 89E L, and wing texture was intermediate between that found in diploids and triploids.

Long interior regions: Using the overlapping translocation method previously described, aneuploidy of a number of long interior regions was studied, as Table 2 shows. Hyperdiploids survived in only two of the experimental crosses, in cases where the portion of the third chromosome carried in excess of 2A was not extremely long. These hyperdiploids, indicated in parenthesis in the body of the table, showed effects of genic imbalance—small eyes and outstretched wings. According to the data in Table 2, hypertriploid females are the most viable long interior region aneuploids. These also often showed signs of aneuploidy—small eyes, misshapen legs, and wings sometimes held apart. Coarse wing texture and large eye facets distinguished these hypertriploids (except in the case of hypertriploids for region 12-89E). Hyperintersexes carrying in excess of 2X3A a long interior region occurred in the progeny of three experimental crosses only. The latter were typically intersexual and showed no shifts toward maleness.

The numerous nondisjunctional forms indicated in Table 2 constitute a surprising feature of the progeny of males heterozygous for two different 3;4 translocations and *y²; ru ca* triploids. If nondisjunction of the two third chromosomes, accompanied by normal disjunction of the two second chromosomes, occurred at meiosis in males heterozygous for two 3;4 translocations, then the resulting sperm would be haploid for chromosome 2 and possess either no third chromosome or two of them. Viable nondisjunctional progeny would result from the union of triploid eggs carrying different numbers of chromosome 3 and of chromosome 2, as indicated in Table 3. Possible nondisjunctional sperm types, triploid eggs, and resulting nondisjunctional progeny appear in this table. These nondisjunctional forms, appearing *y²; y²; ru ca;* and wild type actually occurred in the experimental crosses, as reference to Table 2 will show. Nondisjunction was higher when the breakage points of each of the 3;4 translocations in the male parents were located within a single chromosome arm away from the normal position of the centromere of chromosome 3. More such translocations were available for study in the right arm of chromosome 3. The highest rate of nondisjunction occurred in males heterozygous for T(3;4)85C and T(3;4)A28. To demonstrate that the vigorous, normal appearing *y²* and wild type nondisjunctional forms occurring in the progeny of *ca* T(3;4)85C/ *ru*, T(3;4)A28 males × *y²; ru ca* triploid females were not hypoploids, a control cross of the following composition was made: T(3;4)85C/ *ru ca*, T(3;4)A28 males × *y²; ru ca* triploids. If the *y²* and wild-type progeny of the first cross had been hypoploids, we should expect to find some *ca* "hypoploids" in the progeny of the second cross. No *ca* offspring was derived from the second cross. Hence, the exceptional *y²*, wild-type and *y²; ru ca* progeny of the first cross were assumed to be nondisjunctional forms.

TABLE 2

Aneuploidy involving interior regions of chromosome 3

T(3;4) male parent × y^2; *ru ca* triploid ♀	Overlap hyper-triploid ♀ *ru ca*	Overlap hyper-intersex *ru ca* or y^2; *ru ca*	Nondisjunctional forms except where noted in parenthesis							Control 2X3A intersex *ca*
			Diploid y^2; *ru ca* ♀	♂	Diploid y^2 or wild type ♀	y^2 ♂	Triploid ♀ wild type	Intersex wild type	Intersex y^2	
ca, 13/*ru*, H1	18	3 y^2; *ru ca* 7 *ru ca*	(3 hyper); 9 n.d.	14	2+	1	4	6	2	256
ca, 13/*ru* 89E	1	0	5	10	3 y^2; 4+	1	20	23	1	135
ca, 13/*ru* 28	1	0	2	3	2+	1	3	0	2	188
ca, 85C/*ru*, 2	42	2 *ru ca*	(4 hyper) 7 n.d.	5	12 y^2; 20+	30	49	10	5	321
ca, 85C/*ru*, 28	3	0	17	10	1 y^2; 4+	1	185	241	152	195
ca, 85C/*ru*, 30	13	0	32	19	14 y^2; 17+	10	11	16	5	309
ca, H1/*ru*, c	42	36 y^2; *ru ca* 60 *ru ca*	1	2	1	0	0	0	0	273
ca, c/*ru*, 30	7	0	(1 hyper)	0	3 y^2; 9+	6	13	12	8	147
ca, 85C/*ru*, H5	1	0	0	0	1+	3	63	68	46	120
ca, 12/*ru*, 89E	2*	0	0	0	0	0	33	14	4	171
ca, H3/*ru*, 89E	0	0	17	6	0	1	2	0	0	100
ca, 12/*ru*, 2	1	0	0	0	11 y^2; 13+	16	42	9	3	65

* May be 89E L hyper T.

TABLE 3

Origin of nondisjunctional progeny of wild-type males heterozygous for two 3;4 translocations and y²; ru ca *triploids*

N–d sperm	Triploid egg	N–d progeny	
1X 2 III 1 II	2X 1 III 2 II	3X 3 III 3 II	wild-type triploid
1X 2 III 1 II	1X 1 III 2 II	2X 3 III 3 II	wild-type intersex
Y	1X 2 III 2 II	Y 1X 2 III 2 II	y^2; *ru ca* male
Y	2X 2 III 2 II	Y 2X 2 III 2 II	y^2; *ru ca* female
Y 2 III 1 II	2X 1 III 2 II	Y 2X 3 III 3 II	y^2 intersex
Y 2 III 1 II	1X 1 III 2 II	Y 1X 3 III 3 II	y^2 super-male
X	1X 2 III 2 II	2X 2 III 2 II	wild-type female
X	2X 2 III 2 II	3X 2 III 2 II	wild-type triple-X female
Y 2 III 1 II	1X 1 II	Y 1X 2 III 2 II	y^2 male
Y 2 III 1 II	2X 1 II	Y 2X 2 III 2 II	y^2 female

Among the nondisjunctional diploid forms, some haploidy of chromosome 4 occurred. Thus, some y^2 or wild-type individuals had somewhat thinner bristles than normal, and some wild-type individuals exhibited a strong trident marking. The mutant *ru* showed a pronounced expression in haplo-4 y^2; *ru ca* diploids. Haploidy of chromosome 4 occurred because the maternal triploid strain had been carried a long number of years and was probably diplo-4, as RUDKIN and SCHULTZ (1956) found to be true in their triploid strains carried for a long time.

DISCUSSION

BRIDGE's discovery of 2X3A triploid intersexes in *Drosophila melanogaster* led to his conclusion that male determiners must be present in the large autosomes (BRIDGES 1921, 1922). Succeeding efforts to further locate the autosomal male determiner(s) have proceeded in two directions: (1) by studies of intersex-producing mutants which have occurred in the autosomes of various species of Drosophila, and (2) by diploid and triploid aneuploid studies. GOWEN and FUNG (1957) concluded from the interaction in diploid and triploid combinations of the intersex mutants *Hr*, *tra*, and their normal allele, that the latter is a major sex gene, a view which had been anticipated for this locus by GOLDSCHMIDT (1955). LEBEDEFF (1938) regarded the normal allele of the ix^m mutant he studied in *D. virilis* as a male determining gene, the ix^m mutant being its "stronger allele." SPURWAY and HALDANE (1954) pointed out that if the normal alleles of *ix* mutants are sex genes, then the former must be thought of as hypermorphs rather than hypomorphs according to MULLER's (1932) classification, a conclusion in accordance with that of LEBEDEFF (1938). STONE (1942), working with *D. virilis*, considered the normal allele of Ix^B a sex gene but not necessarily male determining. Earlier authors did not think of the normal alleles of the *ix* mutants as being necessarily sex genes (STURTEVANT 1920; DOBZHANSKY and SPASSKY 1941; WHITE 1948).

Triploid aneuploid studies of both chromosome 2 and 3 in *D. melanogaster* give us no information as to the location of male determining regions within

either of these autosomes (PIPKIN 1947, 1959, and the present work). Hypertriploid females possessing 3X3A plus either the right- or left-hand fragment of two different translocations, T(3;4)85C and T(3;4)89E, showed no intersexual characters. Similarly, no sex shift was found in a number of other different hypertriploid females that in excess of 3X3A carried various long regions, respectively, of chromosome 3 obtained by the overlap method. Long-region hyperintersexes, where these survived, appeared typically intersexual. The normal allele of *Hr* and *tra* is located in the left-hand fragment of T(3;4)85C and of T(3;4)89E. Furthermore, triploid aneuploid studies of chromosome 3 do not confirm the conclusion of KELSTEIN (1938) that a male sex determining region exists between 89A and 94A on the salivary map of chromosome 3, following his study of hypodiploids for this short section.

Since no triploid aneuploids involving any region of chromosome 2 or 3 separately show a sex shift, it is possible that the male determiners responsible for the shift to intersexuality in 2X3A triploid intersexes are located in both the second and third chromosomes. Use of the triploid method to study aneuploidy simultaneously at the locus of L. V. MORGAN's second chromosome intersex mutant and at the locus of the third chromosome *Hr*, *tra* mutants would present the serious technical difficulty of maintaining a sufficiently vigorously triploid strain carrying both second and third chromosome marker mutants. In case such a study is attempted, the sections covering the respective intersex mutant loci should be very short to avoid complications in obtaining aneuploids owing to nondisjunction of one or both pairs of large autosomes in the male parent as well as inviability of the desired aneuploid.

Observations on cell size as determined by facet size and hair spacing in wings (wing texture) in third chromosome triploid aneuploids show that the size of these eye and wing cells is not invariably proportional to the total chromosome content. While most triploid aneuploids show coarse wing texture and large eye facets, the hypertriploid combination 3X3A + 89E L possessed eye facets and wing texture the size of those in diploids. DOBZHANSKY (1929b) also found aneuploid exceptions to the general rule that cell size increases directly with total chromosome content.

SUMMARY

No shift toward intersexuality was observed in hypertriploid females carrying 3X3A + either the right- or left-hand fragments of two different 3;4 translocations, T(3;4)85C and T(3;4)89E. Various other hypertriploid female combinations carrying different long interior regions of chromosome 3, respectively, plus 3X3A showed no intersexual features. Several hyperintersex combinations carrying 2X3A plus a long fragment of chromosome three appeared typically intersexual. Since no sex shift has been observed in triploid aneuploids involving the second chromosome only (PIPKIN 1947) or in triploid aneuploids involving the third chromosome only (PIPKIN 1959 and present study), the suggestion is made that both 2 and 3 chromosome regions may be responsible for the shift toward maleness found in ordinary 2X3A triploid intersexes.

ACKNOWLEDGMENTS

The author is indebted to MR. DAVID W. RAY and MR. PRESLEY AUTRY of Howard University for technical assistance; to DR. E. B. LEWIS, of the California Institute of Technology, for certain 3;4 translocations, unpublished information regarding their breakage points, and for a translation from the Russian of L. V. KELSTEIN's paper.

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GENETIC CONTROL OF DOPA OXIDASE ACTIVITY IN DROSOPHILA MELANOGASTER. I. ANALYSIS OF WILD TYPE, SABLE, SUPPRESSOR-OF-SABLE AND SUPPRESSED SABLE STRAINS[1]

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ADVANCES in the area of biochemical genetics in recent years have provided a number of ideas about primary genetic events and subsequent epigenetic phenomena. These advances have come primarily from studies with microorganisms where the focus has been at the cellular level. Advances have also been made in recent years in the area of population genetics, but to a great exent when the focus is at the population level the underlying biochemical control has been only abstractly considered. The intent of this investigation in its broadest aspects is to bridge these two areas of genetics by analyzing, at the population level, a specific biochemical pattern and its underlying genetic control. The test system selected for analysis is the dopa oxidase system (the primary enzyme believed to be involved in melanin formation) in *Drosophila melanogaster*.

To understand the genetic control of dopa oxidase activity at the organismal level as a stepping stone to analysis at the population level, a study was made of the enzyme system of the classical temperature mutant, sable, and the interacting locus, suppressor-of-sable. This first report deals specifically with analysis of these genotypes. Since a prime aim of the over-all investigation is to uncover different control mechanisms of dopa oxidase activity and determine how they are maintained in populations, analysis of these particular stocks and a wild type for comparison is a good point of departure. This analysis could elucidate a mode of action of suppressor genes and temperature at the biochemical level and thereby contribute to our understanding of the role of such Mendelian and non-Mendelian variables in evolution. In addition to characterizing the dopa oxidase system of the wild type, this study is concerned with the following questions. Is the enzyme system of sable flies grown at lower temperatures different from that of sable flies grown at higher temperatures where the mutant phenotype is not expressed, and is this in turn different from the enzyme system of the wild type? Is the enzyme system of suppressed sable flies like that of the wild type? Does the sable gene control the synthesis of an enzyme altered with respect to a thermal property such as thermolability, thermoactivation or thermoinhibition?

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MATERIAL AND METHODS

Sable (*s*) is a sex-linked recessive mutation; affected individuals are black-bodied. Sable flies grown at 19°C or lower express the mutant phenotype, at 25°C or above are phenotypically wild type, and between these two temperatures have wild type body color but are slightly darker than flies raised at the higher temperature. The suppressor-of-sable gene (*su²-s*), also sex-linked and recessive, inhibits the expression of the sable phenotype. The Canton-S wild type strain is used as a standard for comparison.

Eggs were laid by large numbers of females over short periods of time at 25°C. In the case of flies grown at 15°C, eggs were laid at 25°C and transferred to the lower temperature immediately after the egg-laying period. All experiments were performed on flies raised at 25°C except where specifically indicated otherwise. Comparisons between flies raised at different temperatures are made only between flies having the same parents. All flies were grown in uncrowded cultures on the standard corn meal-molasses-agar medium. Flies are collected within four hours after eclosion, frozen immediately, and stored up to five days at −196°C.

Dopa oxidase extracts are prepared by homogenizing the frozen flies in chilled phosphate buffer (M/15, *p*H 7.0) at a concentration of 50 mg of flies per ml buffer. The homogenate is centrifuged at 0°C for ten minutes at 21,000 g's. The supernatant is then incubated at 0°C during which time the enzyme becomes activated. Dopa oxidase activity is assayed by adding saturating amounts of substrate, 3, 4 DL dihydroxyphenylalanine (dopa) to samples of the extract and measuring the production of dopachrome at 480 mu in a Beckman spectrophotometer, model B. The reaction is carried out at 25°C. Optical density is read at 0.5, 1.0 and 1.5 minutes after addition of the substrate (3.0 ml of 0.02 M dopa in buffer) to 0.1−0.5 ml of extract diluted to 1.0 ml with buffer. Enzyme activity is expressed in terms of dopa oxidase units. One dopa oxidase unit is defined as the amount of enzyme which results in an increase of .001 optical density units per minute. Protein content of the extracts are determined routinely by the method of LOWRY, ROSEBROUGH, FARR, and RANDALL (1951). Activity can be compared either in terms of dopa oxidase units per mg protein or per ml extract since there is no significant variation in the protein content of extracts prepared as outlined above. Large numbers of flies are used in the preparation of each extract randomizing intrastock variability. All measurements have been repeated many times and are reproducible to within ten percent.

RESULTS

Differences in dopa oxidase activity between stocks: The amount of enzyme activity in fully activated extracts is determined by the genotype of the flies used to prepare the extracts. Using flies grown at 15°C, where the sable phenotype is expressed, the rate of dopachrome formation catalyzed by sable extracts is more than twice as great as that catalyzed by Canton-S extracts. To determine whether expression of the sable phenotype is related to a temperature-dependent characteristic of the enzyme system, dopa oxidase activity was measured in extracts

prepared from sable and Canton-S flies grown at 25°C, a condition in which the mutant gene is not phenotypically expressed. The sable extracts again have more than twice as much enzymatic activity as extracts from Canton-S flies. Extracts of both genotypes have less enzymatic activity when the flies are grown at the higher temperature, and the ratios of activity of the two stocks are the same at the two temperatures. Therefore, although the temperature at which the flies are raised affects the dopa oxidase system with respect to the amount of enzyme activity in crude extracts, the temperature mutant sable does not show any temperature-dependent characteristics to distinguish it from Canton-S.

The effect of the *su²-s* gene on dopa oxidase activity was studied by comparing the activities of extracts prepared from genetically suppressed sable flies raised at high and low temperatures with extracts from similarly treated Canton-S and sable flies. The activity of extracts from the *su²-s* stock was also measured as a basis for comparison. These results are shown in Figure 1. The rate of dopachrome formation catalysed by *su²-s s* extracts is greater than that of the *su²-s* extracts which is greater than that of the *s* extract. Extracts prepared from *su²-s s* and *su²-s* flies raised at 15°C have approximately 20 percent more activity than extracts prepared from sibs grown at 25°C, the same temperature effect found in Canton-S and *s* extracts. The rate of reaction in each extract is proportional to the

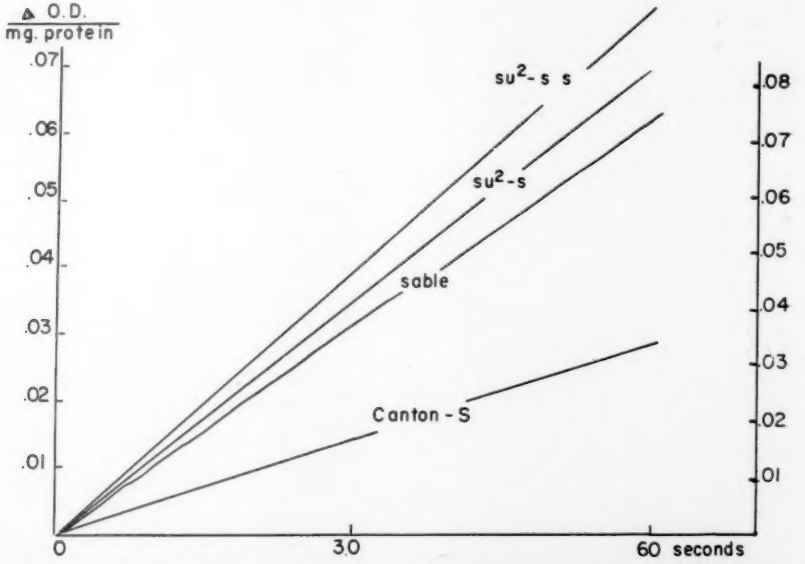

FIGURE 1.—Oxidation of dopa to dopachrome. Relative rates catalyzed by extracts from flies of wild type, sable, suppressor-of-sable and suppressor-of-sable sable genotypes. The left ordinate shows the change in O.D. (480 mμ) in extracts prepared from flies raised at 25°C, and the right ordinate is the scale of the change in O.D./mg protein in extracts prepared from flies raised at 15°C.

enzyme concentration. Assigning the Canton-S extract a value of one, the *s*, *su²-s* and *su²-s s* extracts have relatively 2.2, 2.4 and 2.7 times as much catalytic activity, respectively.

Differences between stocks in dopa oxidase activation: Freshly prepared extracts show no enzyme activity because dopa oxidase is extracted from flies in an inactive form. During a period up to several hours following preparation of the extract, enzymatic activity appears and increases following the kinetics of an autocatalytic process. A major component of the activating system is a protein which will henceforth be referred to as the activator. The kinetics of the activation process of the enzyme from Canton-S flies have been studied by HOROWITZ and FLING (1955), and our data support their proposal that $A + B = C + 2A$ where A is the activator, B is the proenzyme and C is the active enzyme. The dopa oxidase activation is shown in a linear plot in Figure 2 where a is the initial concentration of the activator, b is the initial concentration of the enzyme precursor and x is the concentration of dopa oxidase at time t. Since it is assumed that one molecule of proenzyme gives one molecule of activator and one molecule of active enzyme, the concentration of a, b and x can be calculated in dopa oxidase units. The term $(a + x)$ equals the dopa oxidase activity at time t and the term $(b - x)$

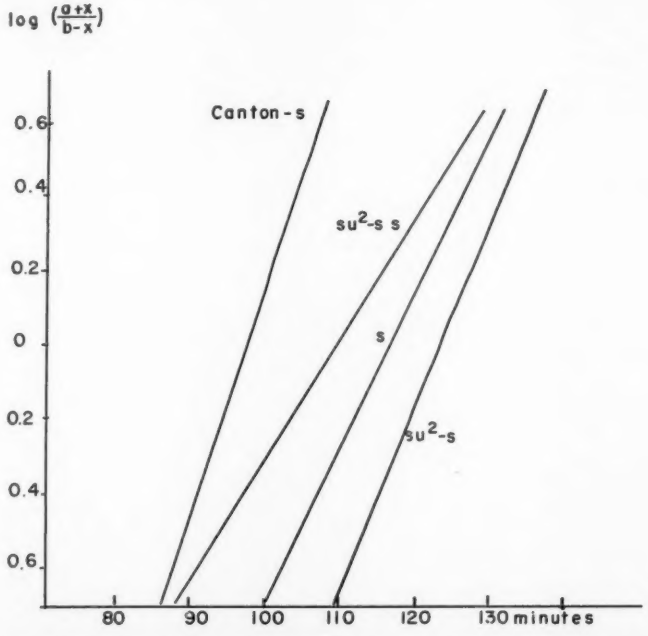

FIGURE 2.—Comparison of the rates of dopa oxidase activation in crude extracts from wild type, sable, suppressor-of-sable and suppressor-of-sable sable flies raised at 25°C. Explanation of the ordinate is given in the text.

equals the dopa oxidase activity obtained after full activation minus the activity at time *t*. The different genotypes can be distinguished from each other by differences in their activation patterns. Canton-S extracts become activated at a faster rate than *s* extracts, while *su*^{s}*-s* and *su*^{s}*-s s* extracts activate at a rate not markedly different from that of the *s* extract. The time required to reach half maximum activity for the Canton-S, *s*, *su*^{s}*-s* and *su*^{s}*-s s* extracts is 98, 117, 123 and 111 minutes, respectively.

Effect of temperature on the dopa oxidase system

The dopa oxidase systems of the different stocks were tested for response to heat to learn how the temperature at which the flies are grown influences the level of enzymatic activity. Similarly, such tests could reveal the mechanism in sable responsible for the temperature dependence of phenotypic expression.

Thermosensitivity of crude extracts: To test whether or not the activating systems of Canton-S, *su*^{s}*-s* or *su*^{s}*-s s* can be distinguished from that of *s* with respect to a thermosensitive property, extracts of each genotype were activated at zero, 15 and 25°C. The usual *in vitro* temperature, 0°C, is the control; 15° and 25° are the temperatures at which the *in vivo* expression of the sable gene is different. Within each genotype aliquots of the same extract were activated at the three temperatures. The temperature at which activation takes place does not affect the activation of extracts of the four genotypes differently. Activation at 15°C, compared to that at 0°C, results in lower maximum activity, greater rate of activation and shorter lag period. Activation at 25°C results in almost complete loss of enzymatic activity. The activation curves for the Canton-S and *s* extracts are shown in Figure 3. These results reveal that the enzyme system is thermolabile *in vitro* even within the physiological temperature range.

Differences in the thermosensitivity of the different dopa oxidase systems may exist which are not detectable in crude extracts. Such differences could involve denaturation by heat of the activator, proenzyme, fully activated enzyme or an inhibitory mechanism. More refined tests of heat sensitivity were therefore performed in which the components of the enzyme system were tested separately over an extended temperature range. At least two mechanisms in the *Drosophila* extracts are concerned with the activation of dopa oxidase. One, associated with the lipid fraction, is nonspecific. Extraction of lipids with chloroform results in a delay of the activation process which can be reversed by adding back the lipid which had been removed or by adding certain foreign lipids. The second is "the" activator, a protein, the removal of which completely stops the activation process. This activating protein is separable from the activated enzyme by centrifugation.

Thermosensitivity of activator: Sensitivity of the activator to heat was tested in the following manner. Fully activated Canton-S, *s*, *su*^{s}*-s* and *su*^{s}*-s s* extracts were centrifuged for 30 minutes at 41,000 × gravity at 0°C, after which all of the dopa oxidase activity was found in the pellet. The supernatant, containing the activator, was divided into aliquots and placed in water baths at the specified temperature for the time of treatment. Fresh extract was prepared from flies of a Samarkand wild type strain, selected as the test system because it is characterized

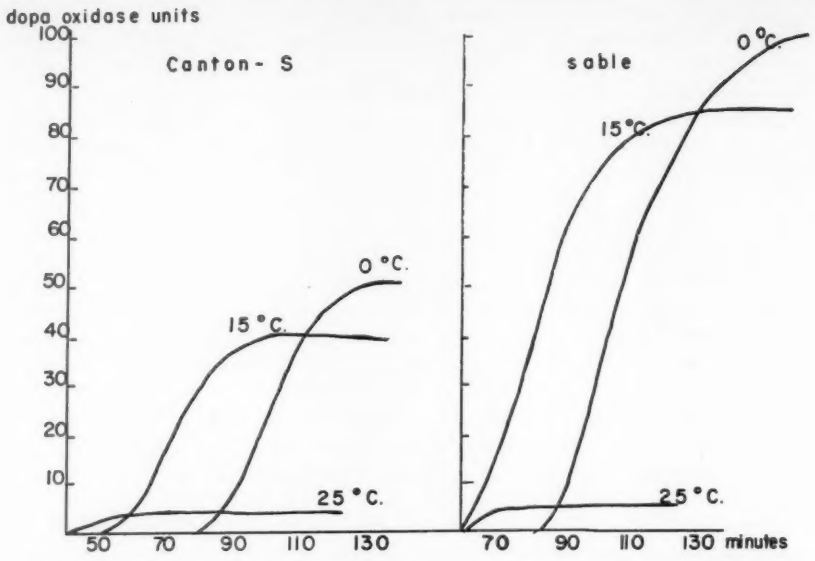

FIGURE 3.—Effect of temperature on the activation process of extracts prepared from Canton-S and sable flies raised at 25°C. Extracts are prepared at 0°C and aliquots are transferred to 15 and 25°C.

TABLE 1

The relative activating capacity of heat-treated and unheated activator of wild type, s, su²-s *and* su²-s s *flies*

Temperature of treatment (°C)	Minutes heated	Activity (percent of control no. 1) at 50 minutes following addition of the activator			
		s	Canton-S	*su²-s*	*su²-s s*
Control no. 1		100	100	100	100
45	30	65	72	71	69
65	2	7	12	8	13
65	20	5	5	4	3
85	2	5	3	4	2
85	20	4	3	3	2
Control no. 2		4	4	2	2

The activating capacity is measured in mixtures of the activator with test extracts. The test extract is a freshly prepared Samarkand extract tested during its normal lag period. Control no. 1 is the mixture to which unheated activator was added and control no. 2 is the mixture to which buffer but no test activator was added.

by a high level of maximum activity. Samples of the temperature-treated activator, unheated activator (control no. 1), and buffer (control no. 2) were added to unactivated test extracts. The measure of functional activator is appearance of dopa oxidase activity in a mixture before it appears in control no. 2. The results are summarized in Table 1. The activating capacity of separated activator kept

at 45°C or less for less than 30 minutes is not significantly altered. It is concluded that thermal denaturation of the activator is not responsible for the loss of activity in crude extracts activated at 25°C and that the activators of the four genotypes tested are not different with respect to their response to heat treatment.

At full activation, test extracts to which heat-treated activator has been added have less activity than the controls. This could be due to the effect of heat on conversion of the activator itself to a competitive inhibitor, or on the release of an otherwise inactive inhibitor. Evidence supporting the latter explanation is given below.

Thermosensitivity of the activated enzyme: The response to heat of the fully activated enzyme of Canton-S, *s*, *su*^{s}-*s* and *su*^{s}-*s s* was tested on dopa oxidase which had been separated from the activator by centrifugation. Aliquots of the activated enzyme were placed for two minutes in a water bath maintained at the elevated temperature, then cooled to 25°C and assayed. The results, shown in Table 2, indicate that with respect to thermostability the dopa oxidases of the different genotypes are indistinguishable. Since thermostability of the partially purified activated enzyme is demonstrated up to 55°C, it is concluded that denaturation of the activated enzyme is not responsible for the loss of activity in crude extracts at lower temperatures.

Thermosensitivity of proenzyme: The comparative sensitivity of the proenzyme to heat was studied in the following experiment. Samarkand activator was separated from the enzyme by centrifugation in the manner described above. This activator (untreated) was added to aliquots of unactivated extracts which had been kept at elevated temperatures for varying lengths of time. The mixtures were incubated at 0°C and assayed for dopa oxidase activity. The results, shown in Table 3, indicate that the proenzyme of each of the genotypes responds similarly to heat.

The temperature sensitivity tests show that decrease in enzyme activity, in crude extracts when activation takes place at temperatures above 0°C up to 25°C, is not due to the direct effect of heat upon the activator or activated enzyme. The possibility that the components of the enzyme system may interact in a manner

TABLE 2

Relative dopa oxidase activity of heated and unheated fully activated enzyme from wild type, s, su^{s}*-s and su*^{s}*-s s flies. The activated enzyme is partially "purified" prior to treatment at the indicated temperature for two minutes*

Temperature of treatment (°C)	Relative dopa oxidase activity (percent of control activity)			
	s	Canton-S	*su*^{s}*-s*	*su*^{s}*-s s*
Unheated (control)	100	100	100	100
45	100	100	103	97
55	100	100	96	103
65	61	63	64	65
75	13	9	16	13
85	1	1	1	1

TABLE 3

The relative activation of heat-treated and unheated proenzyme of wild type, s, su^2-s *and* su^2-s s *flies*

Treatment	Relative dopa oxidase activity (percent of control activity) at 90 minutes after addition of the activator			
	s	Canton-S	*su^2-s*	*su^2-s s*
Unheated (control)	100	100	100	100
5 minutes, 30° C	70	72	75	71
5 minutes, 37°C	37	28	22	23
2 minutes, 45°C	5	5	5	5

Proenzyme activation is measured by adding unheated activator to the proenzyme and incubating the mixture at 0°C to allow activation of the proenzyme. Full activation of each mixture is reached within 90 minutes after addition of the activator.

that makes the integrated whole system reversibly sensitive to heat was considered. Incubation at higher temperatures for varying lengths of time followed by incubation at 0°C showed that inactivation is proportional to the length of time at the higher temperature and is not reversed by subsequent incubation at 0°C. Since the proenzyme in crude extracts has been shown to be very sensitive to heat, being partly destroyed even at 30°C for five minutes, it might be inferred that decreased activity, when crude extracts are incubated above 0°C, is due to lability of the proenzyme. However, it is unlikely that thermal denaturation of proteins occurs at 5, 10 and 15°C. It is more likely that the decreased activity is due to the presence in crude extracts of an inhibitor which is relatively ineffective at 0°C but becomes more effective at higher temperatures. This likelihood is substantiated by the increase, following heating, in the amount of inhibitor in the activator fraction, a fact noted above. A temperature-dependent inactivator has also been proposed by OHNISHI (1959) to explain the effect of temperature on the activation of Musca tyrosinase.

Inhibition studies

Tests were made to determine whether the differences in enzymatic activity and rates of activation between extracts of Canton-S and the other genotypes studied are due to the differential presence of inhibitors.

Inhibition of the oxidation of substrate: That the lower activity in Canton-S extracts is not the result of inhibition of the oxidation of dopa has been shown using two experimental approaches. In the first, samples of Drosophila extracts were added to commercially prepared mushroom tyrosinase (Nutritional Biochemical Co.) to determine whether the added extract decreases activity in the commercial preparation. The results show the presence of an inhibitor in the Drosophila extracts, but the inhibition is the same for each of the extracts. The second method was measurement of the activity in mixtures of fully activated extracts. The presence of a higher concentration of inhibitor in the Canton-S extract would be reflected by a less than additive activity in the mixtures. This is not found.

Inhibition of the activation process: When extracts are dialyzed during the

activation process, the rate of activation increases. That dialysis of extracts removes an inhibitor is shown by the fact that inhibition is affected by adding the concentrated dialysate to an unactivated test extract. Tests show the dialyzable inhibitor is organic and heat stable. This dialyzable inhibitor of the activation process is genotype independent as similar increased rates of activation are found with dialysis in all of the genotypes tested.

Mixing experiments were performed to determine whether Canton-S and sable extracts are distinguishable with respect to inhibitors of activation, other than the dialyzable inhibitor. Activation rates of extracts prepared from Canton-S and sable flies mixed in various proportions were compared with activation rates expected on an additive basis from Canton-S and sable control extracts. There were no inhibitor differences.

Differences in initial concentration of activator

Comparison of the activation curves of Canton-S/sable mixtures with those expected on an additive basis reveals that activity in mixtures is higher than expected throughout most of the activating period (Figure 4). This "enhancement effect" is due to the cross reaction of the activating systems of the two genotypes in the mixture.

It is possible that Canton-S and sable activating systems are qualitatively different and that their interaction in mixtures results in greater efficiency of activation.

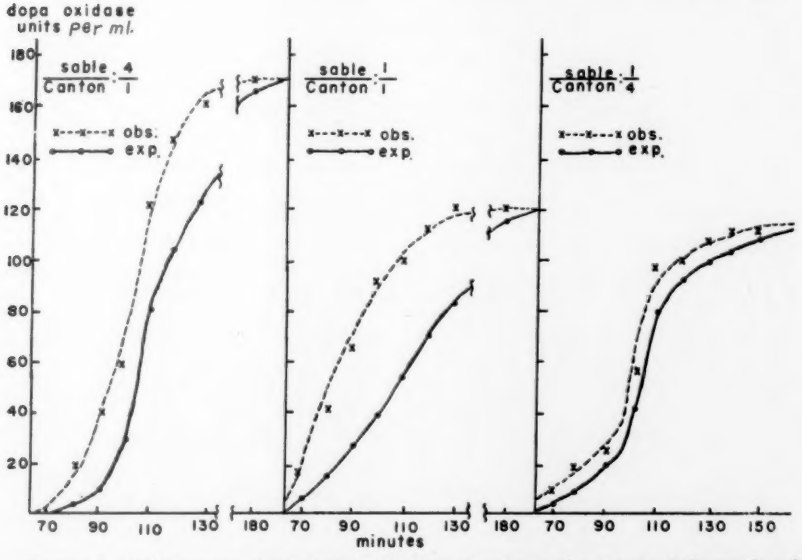

FIGURE 4.—Enhancement effect during activation of extracts of mixtures of Canton-S and sable flies. Expected activity is the sum of activity of Canton-S and sable components calculated from controls.

A more likely explanation for the enhancement phenomenon, however, is that the two activating systems are alike qualitatively, but that Canton-S extracts have a higher initial concentration of activator while sable extracts have a higher initial concentration of proenzyme. (The reasonableness of these assumptions will be discussed later.) Sable proenzyme molecules are activated earlier in a mix than in an extract of sable alone, because of the greater relative number of activator molecules contributed by the Canton-S portion of the mix. This results in the observed enhancement phenomenon. To gather support for this explanation of the enhancement effect, two extracts of sable flies were prepared several minutes apart. Aliquots of the two samples were mixed immediately and activation of the mix was compared with activation of the nonmixed extracts. Activation of the mix in which there are no qualitative differences shows the same enhancement effect as a Canton-S/sable mix. The sable extract which was prepared first had proceeded farther in its activation, and since activator is autocatalytically produced during activation, contributes a higher activator proenzyme ratio to the mix than the second extract. Similar results are obtained if a sable extract is divided into two aliquots, one of which is diluted slightly. The diluted sample activates more slowly and contributes a lower activator/proenzyme ratio to the mix than the undiluted sample.

Dopa oxidase system in Canton-S/sable heterozygotes

Dopa oxidase was studied in Canton-S/sable heterozygotes to determine whether the enzyme system in the heterozygote is like that of either parental strain or like a 1:1 *in vitro* mix of the two enzyme systems. Comparison of the extract from heterozygotes with those of the parental strains is shown in Table 4. The extract from heterozygotes is like that of the Canton-S strain with respect to time of half maximum activity but like the sable stock with respect to amount of activity of the fully activated extract.

Differences in dopa oxidase activity in different lines within stocks

The sable flies used in the experiments reported above were derived from the stock maintained at the University of California. This strain was lost and replaced by a strain derived from the sable stock maintained at the California Institute of

TABLE 4

Comparison of the dopa oxidase systems in crude extracts of Canton-S, sable and Canton-S/sable heterozygotes.

Genotype	t ½ (minutes)	Max. act. (D.U.)
Canton-S	100	125
sable	120	275
Canton-S/sable heterozygotes	100	267

The time of half maximum (t ½) activity is used as a basis for comparing the activation processes. Maximum activity is expressed in dopa oxidase units (D.U.) per ml.

Technology. The dopa oxidase activity in the latter was found to be less than that in the original strain. The dopa oxidase activity of a sable strain derived from a stock maintained at the University of Connecticut was also studied and found to have much higher activity than the other two sable strains. The sable strain obtained from the California Institute of Technology was used to synthesize a new sable suppressed strain. Among the *su*^{s}-*s s* lines derived, lines with different dopa oxidase systems were established. A comparison of the dopa oxidase activity of crude extracts of the different *s* and *su*^{s}-*s s* strains is shown in Table 5. In addition to differences in maximum activity, these strains differ with respect to length of lag period and rate of activation. It is concluded from these findings that the sable and suppressor-of-sable loci, *per se*, have little effect on the dopa oxidase system.

DISCUSSION

One of the aims of this investigation was to determine whether qualitative differences in the dopa oxidase system could be detected in extracts from flies of the four genotypes. The demonstration of qualitative differences would not only make possible deductions about the relationship of the phenotypic effects of the suppressor gene and the temperature mutant to the underlying events at the molecular level, but could also permit the study of the role of specific genes in the integrated pattern of genic action that influence the dopa oxidase system. In addition to the experiments cited above, studies on the *p*H optimum, Q_{10} determinations and substrate profiles were made for the dopa oxidase system from each of the genotypes. These studies give no indication of qualitative differences between the different systems. The results of this investigation, therefore, show that the dopa oxidase system in extracts from flies of the four genotypes studied are distinctly different but only with respect to quantitative aspects.

The discovery of the different levels of dopa oxidase activity in the wild type, *s*, *su*^{s}-*s* and *su*^{s}-*s s* lines permitted several tentative conclusions which were subse-

TABLE 5

The dopa oxidase activity in different lines of sable, suppressor-of-sable and suppressed sable flies

Strain number	Genotype	Source	Max. act. (D.U.)
1	*s*	Univ. of California	275
2	*s*	Cal. Inst. of Techn.	200
3	*s*	Univ. of Connecticut	600
4	*su*^{s}-*s*	Univ. of California	300
5	*su*^{s}-*s*	Cal. Inst. of Techn.	850
6	*su*^{s}-*s s*	strain 1 × strain 4	340
7	*su*^{s}-*s s*	strain 2 × strain 4	510
8	*su*^{s}-*s s*	strain 2 × strain 4	350
9	*su*^{s}-*s s*	strain 2 × strain 4	240
10	*su*^{s}-*s s*	strain 2 × strain 4	250

quently shown to be incorrect. Greater enzymatic activity in sable extracts than in wild type extracts cannot be correlated to greater pigmentation of sable flies because subsequent investigation shows no relation between level of dopa oxidase activity in crude extracts and body color. There are sable strains with high levels of activity and sable strains with low levels of activity, and likewise high and low dopa oxidase activity has been found in strains with wild type body color. Similarly it could be inferred from the early experiments that the mechanism by which the suppressor gene influences dopa oxidase activity interacts with the mechanism controlled by the sable gene. The increased activity in the suppressed sable stock, using the wild type as the base line, is less than the sum of the increased activity in both the suppressor and sable stocks but greater than the increased activity in either. Later when suppressed sable lines were established which have less enzymatic activity than the original sable stock and a sable strain was obtained which has more enzymatic activity than the original suppressed sable stock, it became evident that the sable gene and the suppressor-of-sable gene, *per se*, have little control over the dopa oxidase system.

It is unlikely that the mode of action by which the sable gene produces its phenotypic effect and the mode of action by which the *su^s-s* gene suppresses the expression of the sable phenotype is through an effect on the dopa oxidase system. Although it is possible that *in situ*, or at specific times during development, there are localized enzyme concentrations directly related to phenotype, it is more likely that phenotypic expression is due to secondary genetic phenomena concerning substrate availability. This suggestion is supported by the work of CANTOR (1959). Employing histochemical methods, his study shows that the cuticle of Canton-S third instar larvae has less phenolic compounds than that of *s*, *su^s-s* and *su^s-s s* larvae.

The observation that flies grown at 15°C have approximately 20 percent greater levels of dopa oxidase than flies grown at 25°C is parallel to the situation in Neurospora where strains have been studied that have very little enzymatic activity when cultured at 35°C, but show much more activity when grown at 25°C (HOROWITZ and SHEN 1952). Experiments which will be described in another paper indicate there is no selective advantage to high enzyme activity at low temperatures. The mechanisms of the temperature effect are unknown though experiments have been performed which show that the lower activity in flies grown at higher temperature is not due to the presence of inhibitors which are lacking in extracts from flies grown at lower temperatures. A phenotypic corollary is found in the fact that flies grown in the cold are generally darker than comparable flies grown at higher temperatures, and in mammals melanization is associated with low temperatures in the Himalayan rabbit, guinea pig and Siamese cat. The occurrence of the temperature effect in such widely separated groups as fungi, insects and mammals may be indicative of a general phenomenon. Although there is no evidence for such speculation, it is not impossible that in cells where dopa oxidase is produced, the net synthesis of the enzyme is either increased due to some component of the cell which acts as an inducer at lower temperatures or decreases due to the greater effect of a repressor at higher temperatures. If such

mechanisms of dopa oxidase production exist at the cellular level, it may be possible to study them using microorganisms.

No clues have been uncovered as to the mechanisms which make sable a temperature mutant. Since thermolabile and thermostable forms of tyrosinase are known in Neurospora (HOROWITZ and FLING 1953), it seemed plausible that a temperature mutant could determine a thermolabile form of the enzyme. While this has not been found in our experiments, the possibility remains that differences in thermolability of some factors of the system could be demonstrated in purified preparations.

The phenotype in this study is dopa oxidase activity. In contrast to phenotypes at the morphological level where the number of events leading up to the phenotype evades estimation, a phenotype at the biochemical level is the result of fewer preceding events. Although it is not possible to estimate the number of genes involved, the following factors may contribute to the observed phenotype: repressors, inducers, permeases, the proenzyme, activators and inhibitors. Several of these factors leading to our phenotype can be measured, permitting inferences about their genetic control. On the basis of the experiments on the kinetics of activation performed in our laboratory and by other workers (HOROWITZ and FLING 1955; OHNISHI 1959; KUEMPEL 1959) the sigmoid curve of dopa oxidase activation at 0°C can be interpreted in the following way. The initial concentration of activator is the limiting factor determining the length of the lag period, the higher the concentration the shorter the lag period. The ratio of the initial concentration of activator to the initial concentration of proenzyme determines the slope of the exponential part of the curve, i.e., the rate of activation. The inflection point of the curve, the time of half maximum activity, is the point at which the concentration of the activator and proenzyme are equal. The limiting factor determining the height of the curve is the concentration of the proenzyme. In the experiments where activation was carried out above 0°C, the lag period is shortened and the rate of the reaction increased due to the increased reaction velocity resulting from the increased temperature, and the reduced height of the curve is explained by the presence of a heat-activated inactivator or inhibitor. This interpretation of the activation curve permits us to dissect the phenotype (dopa oxidase activity) into its components the control of which can be independently analyzed at the individual and population levels. In the four original stocks studied the Canton-S stock has the greatest initial concentration of activator and the lowest concentration of proenzyme while the suppressed sable stock has the highest concentration of proenzyme.

The dopa oxidase system observed in the Canton-S/sable heterozygotes is not due to a dominant allele controlling activator concentration contributed by the Canton-S parent and a dominant allele controlling proenzyme concentration contributed by the sable parent. Segregation of such alleles is not found in testcrosses. Experiments to be reported in another paper show length of lag period and maximum activity are multigenically controlled and both the Canton-S and sable stocks have much variability with respect to the genes which influence the dopa oxidase system. The observed time of half maximum activity and the maximum

activity observed in the heterozygote population is the mean value of the segregants of the few parents used in the Canton × sable cross. In a similar manner the intrastock differences found in the sable and suppressed sable stocks can be explained by the chance combination of genetic factors in the few flies selected as progenitors of subcultures or as parents in crosses. Genetic studies of dopa oxidase control in Glomerella (MARKERT 1950), Neurospora (FOX and BURNETT 1959; HOROWITZ and FLING 1953) and guinea pigs (FOSTER 1956) have also found enzymatic activity to be multigenically controlled. If the length of lag period, rate of activation and maximum activity are controlled by independent genetic mechanisms, we would expect to find these parameters independently associated in different genotypes. A study of the dopa oxidase system of a number of stocks, the details of which are presented elsewhere, confirms this expectation.

SUMMARY

This study is concerned with characterizing the dopa oxidase systems of wild type, sable, suppressor-of-sable and suppressed-sable stocks of *Drosophila melanogaster* as a prelude to analysis of the genetic mechanisms controlling the enzyme system in natural populations. The extractable components of the system include dopa oxidase in a zymogen form, the activator (a protein), a lipid-soluble activating factor, a dialyzable inhibitor of activation, an inhibitor of the oxidation of dopa, and a temperature-dependent inhibitor of the enzyme. None of the stocks investigated are alike with respect to the amounts of activator or proenzyme present in the extracts. The sable and suppressor-of-sable genes have very little effect on the dopa oxidase system, and within each stock there is variability over a wide range. Qualitative differences in the enzyme systems of the different genotypes are not found, and there is no correlation between amount of enzymatic activity and body color. It is, therefore, most likely that the modes of action of the sable and suppressor-of-sable genes are through secondary genetic phenomena rather than through a direct control of the enzyme system.

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LINKAGE ANALYSIS WITH VERY HIGH FREQUENCY MALES OF ESCHERICHIA COLI[1]

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CELL conjugation in *Escherichia coli* permits the oriented transfer of a specific linear array of genetic determinants from an Hfr, or male, donor cell to an F^-, or female, recipient cell (WOLLMAN and JACOB 1955). The sequence of marker transfer may vary from one Hfr type to another; moreover, any single Hfr type is capable of transferring only a fractional part of its entire genetic complement to recipient cells at high frequency. For example, six different Hfr types have been reported by JACOB and WOLLMAN (1957), each of which transfers a different chromosomal segment at high frequency. The discovery of this variety of Hfr types led JACOB and WOLLMAN to propose that the genetic markers of the original F^+ strain which gave rise to the Hfr males are disposed on a closed circle (Figure 1). The chromosome of any single Hfr type can then be described by breaking the circle at one point and designating as "origin" the free end which first penetrates the F^- during conjugation. The details of mating type determination and the role of the sex factor, F, in this process have been described elsewhere (WOLLMAN and JACOB 1958a; ADELBERG and BURNS 1960).

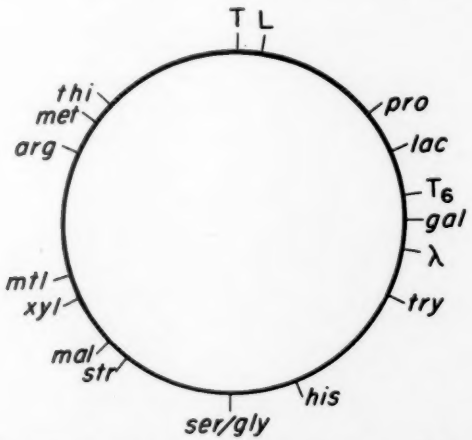

FIGURE 1.—The genetic map of *Escherichia coli* K-12 (after JACOB and WOLLMAN 1958a). Gene symbols are defined in Table 1.

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In this paper we describe three new Hfr mutants which possess the useful property of transferring all of the known genetic markers of *E. coli* to female cells at relatively high frequency. We shall also present additional evidence which these strains have provided in support of the circular linkage group hypothesis.

MATERIALS AND METHODS

Media and mating conditions: Media for the cultivation of parent cultures and for the selection or scoring of genetic recombinants, as well as the details of the standard conditions for Hfr × F⁻ crosses, have been fully described in a previous communication (ADELBERG and BURNS 1960). In all cases, mating mixtures were sampled and plated for recombinant selection at 90 minutes. Adenine, when required in synthetic media, was used at a concentration of 20 μg per ml.

Strains: The strains referred to in this paper are listed in Table 1. They are all originally derived from strain K-12 of *E. coli*.

Selection of Hfr mutants: The replica plating technique of LEDERBERG and LEDERBERG (1952) has been used in the manner to be described to facilitate the detection of rare Hfr mutants in a population of F+ cells (JACOB and WOLLMAN 1956). Approximately 10^7 washed cells from an exponential broth culture of an F+ strain were spread uniformly over the surface of minimal agar plates containing glucose plus growth factors required by the strain. The cells were then exposed to ultraviolet irradiation at doses sufficient to allow 300 to 600 colony-forming survivors per plate. After 48 hours of incubation, at which time surviving colonies were an average 1.5 mm in diameter, they were replicated onto two identical plates of selective medium in which 2×10^9 F⁻ cells had been spread over the agar surface, and onto a third control plate of selective medium without F⁻ cells. With rare exceptions, only mutant Hfr colonies gave rise to congruent sites of recombinant growth on the two test plates. F+ colonies, on the other hand, occasionally gave rise to recombinants on only one plate, but more often yielded no recombinants at all. The Hfr mutants were then easily isolated from the master plates by locating visually those colonies corresponding to the congruent sites of growth on the test plates.

RESULTS

Isolation and characterization of three new Hfr types: We wished to select for Hfr mutants which would transfer the *mal–str–his* region at high frequency, since no such strains had previously been described. Accordingly, UV irradiated master plates, containing surviving colonies of strain W–208 F+ (*mal*+, *his*+, TL⁻), were replicated onto test plates of strain AB–57 F⁻ (*mal*⁻, *his*⁻, TL+) on a medium selective for *mal*+ *his*+ TL+ recombinants. This procedure ultimately yielded three independent mutants which scored as Hfr males. These were purified by two successive colony reisolations from plates streaked with dilute cell suspensions, and assigned the strain numbers AB–311, AB–312, and AB–313. At this time, all three appeared to be stable Hfr males, but later experiments indicated that strain AB–311 spontaneously reverts to the F+ state at a moderate rate and must, therefore, be periodically reisolated.

TABLE 1

Bacterial strains

Strain no.	Auxotrophic characters* TL	*pro*	*try*	*his*	*ser/gly*	*ade*	*arg*	*met*	Energy-source utilization *gal*	*mal*	*xyl*	*mtl*	Response to phages and streptomycin λ	T6	*str*	Sex
W–208 F*	−	+	+	+	+	+	+	+	+	+	+	+	(λ)	S	R	♂
AB–6	+	+	+	−	+	+	+	−	−	−	−	−	R	S	R	♀
AB–57	+	−	+	−	+	+	+	−	−	−	−	−	R	S	R	♀
AB–311	−	+	+	+	+	+	+	+	+	+	+	+	S	S	R	♂
AB–312	−	+	+	+	+	+	+	+	+	+	+	+	S	S	R	♂
AB–313	−	+	+	+	+	+	+	+	+	+	+	+	(λ)	S	R	♂
AB–320	+	−	+	+	+	+	+	−	−	+	+	−	S	S	R	♂
AB–325	+	+	+	−	−	+	−	+	−	−	−	−	R	R	R	♀
AB–331	−	+	+	−	+	+	−	+	−	−	−	−	R	R	R	♀
AB–334	+	+	−	−	+	+	−	+	−	−	−	−	R	R	R	♀
AB–336	+	+	−	+	+	−	−	+	−	−	−	−	R	R	R	♀
AB–359	+	+	+	−	+	−	−	+	+	−	−	−	R	R	R	♀
AB–531	+	−	+	−	+	+	+	−	−	−	−	−	R	R	R	♀

* The following abbreviations are used: TL—threonine and leucine; *pro*—proline; *try*—tryptophan; *his*—histidine; *ser/gly*—serine or glycine; *ade*—adenine; *arg*—arginine; *met*—methionine; *gal*—galactose; *mal*—maltose; *xyl*—xylose; *mtl*—mannitol; *str*—streptomycin; R—resistance; S—sensitivity; (λ)—lysogenic for λ.

A first approximation of the genetic segments transferred at high frequency by the three new Hfr types was obtained by crossing each male to a multiply deficient female strain under standard conditions and determining the recombination frequencies for as many Hfr markers as possible. The results are given in Table 2. JACOB and WOLLMAN (1958) have shown that the occurrence of spontaneous interruptions during the course of oriented chromosome transfer results in a gradient of recombination frequencies, so that early markers exhibit high rates while the last markers transferred generally exhibit very low rates; hence, the order of genetic transfer for a given Hfr is determined by arranging the selected markers according to their recombination frequencies. The data in Table 2 show that of the three strains, only AB–313 has the expected order of genetic transfer; in AB–311 and AB–312, *mal* and *his* are at opposite ends of the transfer sequence.

Additional crosses with appropriately marked F⁻ strains have shown that strain AB–312 transfers the sequence O–*str–mal*–TL–*try–his–ade–ser/gly* at high frequency, where "O" represents origin, while AB–311 transfers O–*his–try*–LT–*mal–str–ser/gly–ade*. The relationship between these sequences and the circular chromosome model of JACOB and WOLLMAN is diagrammed in Figure 2. The recombination rates for the most distal markers of these strains are on the order of one or two percent; thus, they transfer the entire Hfr genome with extraordinary efficiency in comparison to previously described males whose recombination rates for terminal markers generally range from 0.001 to 0.01 percent. We shall henceforth refer to these donors as *very high frequency* (Vhf) males to distinguish them from the usual Hfr strains. The interesting possibility that this behaviour could result from an unusual mode of genetic transfer prompted us to investigate their kinetics of chromosome transfer.

Interrupted mating experiments: WOLLMAN and JACOB (1955) have developed the technique of interrupting chromosome transfer during mating by shearing apart conjugating cells in a Waring blendor, thereby permitting them to follow the progressive transfer of genetic markers into female cells as a function of time. Although this method has proved quite satisfactory for experiments with normal

TABLE 2

Recombination frequencies for Hfr strains AB-311, AB-312, and AB-313

AB-311 ♂ × AB-6 ♀		AB-312 ♂ × AB-57 ♀		AB-313 ♂ × AB-336 ♀	
Selected marker	Recombination frequency	Selected marker	Recombination frequency	Selected marker	Recombination frequency
his⁺	42	*mal*⁺	40	*mtl*⁺	49
gal⁺	12	*xyl*⁺	26	*xyl*⁺	43
met⁺	4	*mtl*⁺	25	*mal*⁺	32
mtl⁺	3.7	*met*⁺	22	*ade*⁺	15
xyl⁺	2.8	*pro*⁺	8	*try*⁺	6
mal⁺	1.5	*gal*⁺	4	*arg*⁺	0.3
		his⁺	2.5		

The indicated crosses were carried out under standard conditions for 90 minutes. Hfr parents were contraselected by omitting threonine and leucine from the selective media. Recombination frequencies are expressed as the number of recombinants recovered per 100 Hfr cells in the mating mixture.

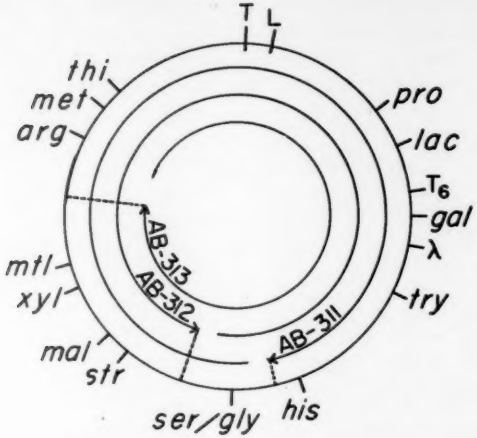

FIGURE 2.—Diagrammatic representation of the chromosome segments transferred at high frequency by strains AB-311, AB-312, and AB-313. Each broken circle represents the linear chromosome of the Hfr strain indicated, and the arrows show the direction of chromosome penetration during conjugation.

Hfr strains, it does not provide consistently reliable data for the Vhf males. On the other hand, the method of interrupting mating by killing the male parent with virulent phage T6 (HAYES 1957) was found to give excellent results. Accordingly, the kinetics of genetic transfer were determined by removing samples at intervals from a mating mixture of T6 sensitive males and T6 resistant females, treating the samples with phage as described by HAYES, and diluting and plating on a variety of selective media.

The results of such an experiment for a cross of AB–312♂ × AB–531♀ are given in Figure 3A, where numbers of recombinants recovered are plotted as a function of the time at which mating is interrupted. The results are essentially analogous to the kinetics observed with Hfr H (WOLLMAN and JACOB 1955), in that there is a steady rise with time of the number of zygotes having received a given Hfr marker prior to interruption. Moreover, the order of gene transfer is seen to be the same as that inferred from the gradient of recombination rates given in Table 2. The intercepts of the curves with the time axis correspond to the times at which the various male markers first penetrate into the zygotes during conjugation; referring to Figure 3A, the *mal* locus of strain AB–312 first enters at 23 minutes after mating begins, the *xyl* locus at 30 minutes, *mtl* at 31.5 minutes, and *met* at 40 minutes. Figure 3B illustrates the identical experiment for a cross of AB–320♂ × AB–331♀; strain AB–320 is a recombinant of AB–312 which has the same "origin" as its parent and is also a Vhf male. Since AB–320 is T^+ and L^+, it was possible to select for these two very closely linked markers, but it was not possible to resolve the data points into two distinct curves; hence, the time of entry for both is given as 50 minutes in Figure 3B.

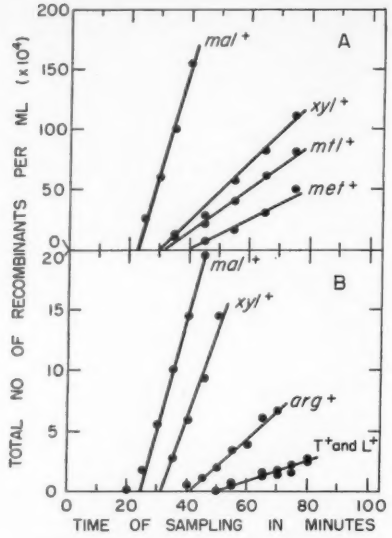

FIGURE 3.—Kinetics of chromosome transfer by donor strains AB-312 and AB-320. A. Kinetics for a cross of AB-312 × AB-531 as determined by periodic interruption of mating with phage T6, followed by plating on media selective for *mal*⁺, *xyl*⁺, *mtl*⁺, and *met*⁺ recombinants. T6-resistant mutants of the donor were contraselected by omitting threonine and leucine from the selective media. B. Kinetics for a similar cross of AB-320 × AB-331. T6-resistant mutants of the donor were contraselected by omitting proline from the selective media.

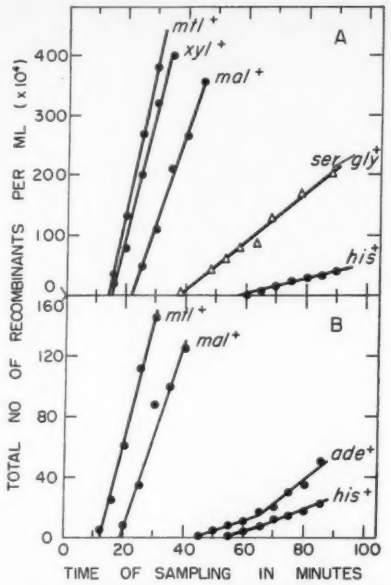

FIGURE 4.—Kinetics of chromosome transfer by strain AB-313. A. Solid circles represent data from a cross of AB-313 × AB-531; triangles are from a cross of AB-313 × AB-325. Conjugation was interrupted with phage T6, and phage-resistant mutants of AB-313 were contraselected by omitting threonine and leucine from the selective media. B. Kinetics for a similar cross of AB-313 × AB-359. The reason for the breaks in the slopes of the curves for *ade* and *his* is not known.

Similar experiments were performed with strains AB–313 and AB–311, as shown in Figures 4 and 5, respectively. Figure 4 includes data from three separate crosses of AB–313 because different F⁻ strains were required for the selection of *ade*⁺ and *ser/gly*⁺ recombinants. (The *ade*⁻ mutant of strain AB–359 was isolated in this laboratory by the agar plate penicillin selection technique (ADELBERG and MYERS 1953)). It should be noted that the *mtl* and *mal* loci are separated in time by an interval of about eight minutes when transferred by either AB–312 or AB–313; this suggests that the two donors transfer their genetic material at similar rates, and that time of entry data from both may therefore be justifiably compared.

WOLLMAN and JACOB (1958) have summarized the evidence which suggests that chromosome transfer proceeds at a constant rate for at least the first half of the entire chromosome length, and they have shown how this constant rate allows

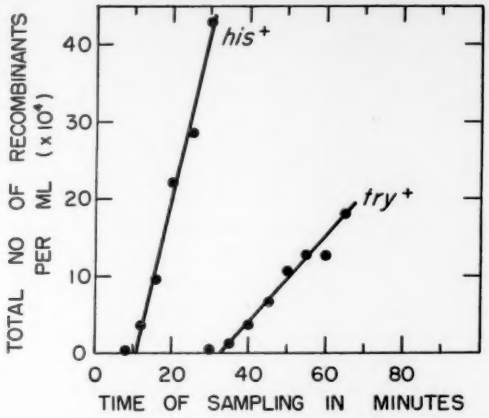

FIGURE 5.—Kinetics of transfer of the *his* and *try* loci by strain AB-311. The F⁻ parent was strain AB-334. Phage T6 interruption and threonine-leucine contraselections were employed.

TABLE 3

Summary of data from time of entry experiments

Male parent	Markers from male parent	Observed distances between markers (in minutes)	Average map distance (in minutes)
AB-312	*mal–xyl*	6, 7	6.5
AB-312	*xyl–mtl*	0.8, 1.6	1.4
AB-312	*xyl-met*	9.5, 11, 10	10.2
AB-320	*xyl-arg*	8.5, 9	8.7
AB-320	*arg*-T	9.5, 8.5	9
AB-313	*mtl–xyl*	1, 1.5, 1	1.2
AB-313	*xyl–mal*	6.5, 7, 6.5, 6.5	6.6
AB-313	*mal–ade*	25.5, 26, 24.5	25.3
AB-313	*xyl–his*	43, 44, 45, 40	43
AB-313	*ser/gly–his*	20, 20	20
AB-311	*his–try*	24, 25, 26	25

one to construct a genetic map by expressing the distance between loci in terms of the time required for their transfer during conjugation. The results of several kinetic experiments with the Vhf males are given in Table 3, along with the average map distances for markers on the chromosome segment extending from TL through *mal* to *try*. The segment covers a total span of 86 minutes; since the distance from *try* through *gal* to TL has been shown by JACOB and WOLLMAN to be 25 minutes, the genetic map of *E. coli* totals 111 minutes in length. This is in general agreement with the only published Hfr map available, which assigns a total distance of 115 minutes for the region TL–*try–mal–met* (WOLLMAN and JACOB 1958).

Previously published maps of the *E. coli* linkage group have been primarily intended to demonstrate relative positions of markers rather than map distances, with the result that the latter feature has often been distorted; consequently, a corrected representation, based upon determinations of times of entry, is given in Figure 6.

Demonstration of linkage with strains AB–311 and AB–312: Once the sequence of genetic transfer for a given Hfr has been determined by interrupted mating and recombination experiments, it becomes relatively easy to demonstrate genetic linkage of the various Hfr markers to one another. Thus, any unselected Hfr marker "A" may be inferred to be linked to Hfr marker "B" if recombinants which receive "B" have a greater probability of receiving "A" than recombinants which do not receive "B". To consider a specific example, Table 4 shows that in a cross of AB–312 δ with an appropriate F^- strain, 51 percent of recombinants selected for *mal*+ carry *mtl*+ as an unselected marker, whereas 89 percent of *xyl*+ recombinants carry *mtl*+ as an unselected marker. The increased probability of receiving the *mtl*+ marker due to *xyl*+ selection clearly indicates that the two loci are linked.

Table 4 summarizes the frequency of unselected markers in recombinants formed when various markers are selected from AB–312 or its derivative, AB–320. When these data are analyzed in the manner demonstrated above, it is found that all of the markers from *xyl* through *try* to *ade* are linked to one another in a single continuous linkage group. The minimum number of data needed to establish this have been extracted from Table 4 and presented in the first half of Table 5.

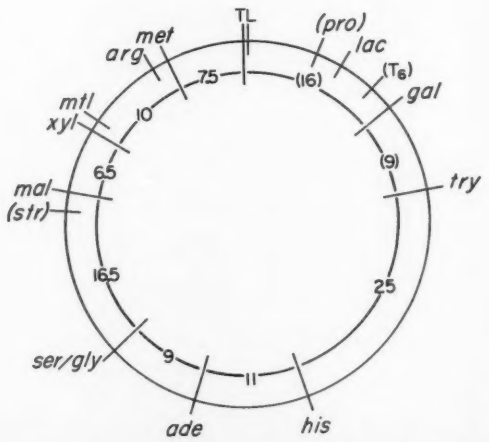

FIGURE 6.—Corrected genetic map of *Escherichia coli*, showing the relative distances between markers as determined by kinetic experiments. Numbers refer to map distances in minutes. Numbers in parentheses are from the data of WOLLMAN and JACOB (1958) for strain Hfr H. Markers in parentheses have not been precisely mapped.

TABLE 4

The frequency of occurrence of unselected markers in several classes of recombinants selected from crosses with donor strains AB-312 and AB-320

Selected recombinants*	No. of colonies scored	Frequency of unselected male markers in percent								
		mal	*xyl*	*mtl*	*arg*	T	L	*try*	*his*	*ade*
mal$^+$	120	..	54	51	32	..	..	18	4	2
xyl$^+$	120	91	..	89	56	25	18	16	5	7
mtl$^+$	120	82	94	..	57	..	..	32	7	5
arg$^+$	120	83	79	80	..	55	50	68	27	17
L$^+$	80	..	85		82	93	..	..	25	..
try$^+$	120	85	83	85	80	..	..	..	55	31
his$^+$	120	84	81	80	80	..	..	71	..	54
ade$^+$	80	80	71	71	55	..	..	62	77	..

* Recombinant selections were made from standard 90 minute matings. Threonine-leucine contraselections were used for AB-312 crosses, and proline contraselections were used for AB-320.

TABLE 5

Analysis of linkage in donor strains AB-312 and AB-311

Parents	Selected recombinants*		Unselected marker (U)	Ratios		Indicated linkage
	(S_1)	(S_2)		U/S_1	U/S_2	
AB-312 ♂ × AB-336 ♀	*mal*$^+$	*xyl*$^+$	*mtl*$^+$	0.51	0.89	*xyl–mtl*
AB-312 ♂ × AB-336 ♀	*mal*$^+$	*mtl*$^+$	*arg*$^+$	0.32	0.57	*mtl–arg*
AB-312 ♂ × AB-336 ♀	*mal*$^+$	*arg*$^+$	*try*$^+$	0.18	0.68	*arg–try*
AB-312 ♂ × AB-336 ♀	*mtl*$^+$	*try*$^+$	*ade*$^+$	0.05	0.31	*try–ade*
AB-311 ♂ × AB-336 ♀	*arg*$^+$	*mal*$^+$	*ade*$^+$	0.11	0.37	*ade–mal*
AB-311 ♂ × AB-336 ♀	*try*$^+$	*arg*$^+$	*mal*$^+$	0.025	0.20	*mal–arg*

* Recombinant selections were made from standard 90 minute matings, and threonine-leucine contraselections were employed against both donor strains. One hundred and twenty colonies of each class of selected recombinants were scored for the indicated unselected markers.

To confirm the circular linkage group proposed by JACOB and WOLLMAN for the F+ ancestor of these Hfr strains, it only remains to demonstrate linkage of the markers *ade–ser/gly–mal–xyl*. This cannot be done with strain AB–312 but is readily accomplished with Vhf strain AB–311. The necessary data are given in the second half of Table 5 in which the linkage of *ade* to *mal* and of *mal* to *arg* is established. The combined data from strains AB–312 and AB–311 thus provide conclusive proof of the continuous linkage of all the known chromosomal markers of *E. coli* K–12. These results fully substantiate JACOB and WOLLMAN's theory of the closed (circular) linkage group for the F+ progenitors of the various known Hfr types.

DISCUSSION

Numerous attempts to determine the complete genetic map of *E. coli* have been made. CLOWES and ROWLEY (1954) were able to place all of the known markers of strain K–12 on a rectilinear chromosome by applying the test for linkage described

in the preceding section to the recombinants issuing from $F^+ \times F^-$ crosses. JACOB and WOLLMAN have stated that all of the known markers are linked together on a circular structure, on the basis of the different sequences of markers transferred by different Hfr strains. Many of the inferred sequences, however, are based on relative recombination frequencies and on times of entry as determined by interrupted mating experiments; such data do not constitute evidence of linkage. For example, male strains of *E. coli* have been isolated which transfer both sex factor (F) and chromosome independently (JACOB and ADELBERG 1959; ADELBERG and BURNS 1960). One such strain transfers F at five minutes with a frequency of 90 percent, and TL at 20 minutes with a frequency of about one percent. However, F and TL are not linked.

JACOB and WOLLMAN (1958) presented true linkage data for the region TL–*his–mal* based on crosses with strain Hfr H; evidence for linkage from *mal* through *arg* to TL, however, has not previously been published. The missing data are now available in Tables 4 and 5, and the final corrected map is presented in Figure 6. An interesting feature of the corrected map is the revelation that all of the earlier work on linkage was based on markers covering only one third of the total chromosome; i.e., the region *str*–TL–*gal*.

The interrupted mating experiments reported here indicate that the mechanism of chromosome transfer in the Vhf males does not differ in any recognizable way from the mechanism in ordinary Hfr strains. In both instances, the chromosome is transferred with a specific orientation, and with a speed which permits the entire chromosome to be transferred in about two hours under standard conditions. Presumably, the high recombination rates for distal markers are the result of very low rates of spontaneous chromosome rupture during the course of conjugation. The formation of particularly stable conjugation bridges on the part of the male cells may also contribute to the high recombination rates. However, there is no evidence available at this time to permit a choice between these explanations. It is of interest in this connection that ANDERSON (1958) has observed only limited chromosome transfer between conjugants which remained visibly joined for two or more hours, suggesting that it may be the breakage of the chromosome rather than of the cytoplasmic connection which halts the transfer process.

Whatever the basis of their prolonged chromosome transfer may be, the Vhf males are particularly useful for the mapping of new mutant loci. Regardless of where on the chromosome the mutation has occurred, a single cross with a Vhf male suffices to locate it with relative precision. Furthermore, these strains make accessible to mapping the entire *mal–ade–try* region, which is transferred only at relatively low frequency by all previously described males.

SUMMARY

Three new Hfr (male) strains of *Escherichia coli*, which possess the unique property of transferring the entire male genome to females at relatively high frequency, have been described. The term Vhf (very high frequency) male is proposed to distinguish these from ordinary Hfr strains.

Studies with the Vhf males have provided recombination and linkage data which fully confirm the JACOB-WOLLMAN theory of a closed (circular) linkage group for *E. coli* K–12. The use of the Vhf males for the precise mapping of markers along the entire chromosome, as well as for the rapid localization of new mutant loci, is illustrated and discussed.

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EVIDENCE OF NON RANDOM DISTRIBUTION OF ASCUS CLASSES IN FRUITING BODIES OF NEUROSPORA CRASSA

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THE observation of morphologically identical twins among immature and degenerate asci (MITCHELL 1959) has suggested that unpredicted genetic relationships may exist between adjacent asci and may be reflected by similarities in phenotypes of strains obtained by serial isolations of ascospores from mature asci. Subsequent genetic analyses have indicated that adjacently formed asci may, rather regularly, show similar or identical patterns of segregation of known markers. This paper reports further indication of such a relationship provided by the results of analysis of individual perithecia from an exceptionally fertile cross.

A second motive for the work to be described stems from an interest in ratios of ascus classes. The observation of preferred recombinant frequencies among random spores (MITCHELL and MITCHELL 1954; MITCHELL 1958) leads to the prediction that preferred ratios of segregation classes among asci occur and, indeed, some indication of such preference may be seen in existing ascus data (MITCHELL 1960). However, larger numbers of asci per cross are needed, particularly if linked markers are involved.

A third point to be discussed, concerning the order of spore pairs within the ascus, is raised by the data presented and has been considered earlier by CATCHESIDE (1944) and WHITEHOUSE and HALDANE (1945).

METHODS

The cross: The two markers followed are *pyr-3a* (pyrimidine, 37301) and *cot* (colonial, temperature-sensitive, C 102), both assigned to linkage group IV. Parents of the cross considered are not standard strains but segregants from an ascus produced by an earlier cross of *cot* × *pyr*. This ascus came from the only exceptionally fertile perithecium among about 30 examined. The *pyr* parent (37301-3250-4A) of the earlier cross has been used as a standard strain for several years. The *cot* parent, on the other hand, is a recombinant from a cross between two very closely linked markers of group I, with one parent expressing *cot*. Three generations of inbreeding, with the latter three markers present, had preceded isolation of the *cot* recombinant but, since it is not known that inbreeding affected the results to be reported, details will not be given.

The cross, *cot*-8025-1*a* × *pyr*-8025-7*A*, was made on unsupplemented WESTERGAARD and MITCHELL medium, with *cot* as protoperithecial parent, and left to

mature at room temperature (about 24°C) for 22 days from the time of "fertilization".

Dissection of asci: Ascus dissections were done on shallow plates containing about 20 ml of nitrate minimal medium (STRAUSS 1951) solidified with four percent agar. In the center region of the plate an ascus cluster was removed from its perithecium by first pinching a hole in the perithecial wall with sharp forceps and then squeezing the contents into a drop of water. As those who dissect Neurospora asci are well aware, the walls of mature asci are fragile, and it is therefore somewhat difficult to free the asci from the perithecium without rupturing them. The rather unusual thinness and softness of the walls of perithecia from this cross aided in the attempt to obtain the ascus cluster as nearly intact as possible. The spores of individual asci were then separated by moving them outward, serially, towards the border of the plate. The intention was to dissect adjacent asci consecutively, so that the positions of dissected asci on the plate would bear a relationship as close as could be obtained to their positions in the ascus cluster. However, since the cluster is more like a filled circle than a ring, exact duplication could not be realized. Also it was estimated that, at best, not more than a third to a half of the normal-appearing asci per cluster were tested.

Classification of segregants: The plates of spores were heat-treated at 60°C for 30 minutes and then incubated at room temperature (about 24°C) overnight. After this, two classes of segregants were scored under a dissection microscope. Spores that had produced only very short germ tubes were classified as *pyr* and those that had given rise to small "colonies" consisting of several infrequently branched hyphae, as *pyr*+. If no germ tubes or hyphae had been produced the spore was, of course, scored as ungerminated. About 1 ml of a uridine solution (1 mg/ml) was then poured on the plate and spread over the "colonies" by tilting. This rarely disturbs the positions of the germinated spores. During the next five hours at 34°C water from the uridine solution was absorbed or evaporated; the *pyr* segregants grew in response to uridine and the expression of *cot* appeared. The four types of segregants then scored were characterized as follows: ++—hyphae continue to elongate at 34°C and branch infrequently; + *cot*—hyphae do not elongate at 34°C but produce frequent, regularly spaced, short side branches; *pyr* +—one or more of the short germ tubes elongate to form hyphae which branch infrequently; *pyr cot*—the short germ tubes branch laterally to form a tiny, compact "colony".

DISCUSSION OF RESULTS

Tabulation of data: The numbers of asci of each class from each of the nine perithecia tested are shown in Table 1. The phenotypes are designated as follows: r = *pyr* +; s = + *cot*; rs = *pyr cot*; + = + +. All asci are included from which as many as five of the eight spores germinated and could be scored. This insures that at least one member of each of three of the four spore pairs of an ascus will be represented. Actually, germination was exceptionally good. From 282 asci scored only about nine percent of the spores failed to germinate; thus few had as many as three ungerminated spores. The classifications made are, there-

TABLE 1

Number of asci of each class from each of nine perithecia from + cot × pyr +. *Phenotype designations are:* r = pyr +; s = + cot; rs = pyr cot; + = + +

	Ascus classes	Phenotypes of spore pairs 1, 2	3, 4	5, 6	7, 8	Ascus classes per perithecium 1	2	3	4	5	6	7	8	9	Totals	
PD-1	1	r	r	s	s	3	8	8	9	9	7	10	12	12	78	157
	2	s	s	r	r	.	7	9	7	13	9	13	7	14	79	
PD-2	1	r	s	r	s	.	1	1	3	.	4	1	2	2	14	52
	2	s	r	s	r	1	.	1	1	3	2	2	2	3	15	
	3	r	s	s	r	2	.	1	2	2	1	.	2	2	12	
	4	s	r	r	s	1	.	.	2	2	2	1	1	2	11	
T-1	1	r	rs	+	s	.	.	4	1	.	.	1	.	1	7	59
	2	rs	r	s	+	.	.	1	1	1	1	2	2	1	9	
	3	r	rs	s	+	.	.	.	1	.	1	1	.	1	4	
	4	rs	r	+	s	.	.	.	.	1	1	1	1	.	4	
	5	+	s	r	rs	.	.	.	.	3	1	1	.	1	6	
	6	s	+	rs	r	.	.	2	2	1	1	1	1	.	8	
	7	+	s	rs	r	.	.	1	3	2	1	1	.	4	12	
	8	s	+	r	rs	.	.	2	.	1	1	1	2	2	9	
T-2	1	s	rs	+	r	.	.	.	.	.	.	.	.	.	..	6
	2	rs	s	r	+	.	.	.	.	.	.	.	.	.	..	
	3	s	rs	r	+	.	.	.	.	.	.	.	.	.	..	
	4	rs	s	+	r	.	.	.	.	.	.	.	.	.	..	
	5	+	r	s	rs	.	.	.	.	1	.	1	2	1	5	
	6	r	+	rs	s	.	.	.	.	.	.	.	.	.	..	
	7	+	r	rs	s	.	.	.	.	.	.	.	.	1	1	
	8	r	+	s	rs	.	.	.	.	.	.	.	.	.	..	
T-3	1	r	s	rs	+	.	.	.	.	.	.	.	.	.	..	8
	2	s	r	+	rs	.	.	.	1	.	.	.	.	.	1	
	3	r	s	+	rs	.	.	.	.	1	.	1	1	.	3	
	4	s	r	rs	+	2	.	.	.	.	.	.	.	.	2	
	5	rs	+	r	s	.	.	.	.	.	.	.	.	.	..	
	6	+	rs	s	r	.	.	.	.	.	.	.	1	.	1	
	7	rs	+	s	r	.	.	.	.	.	1	.	.	.	1	
	8	+	rs	r	s	.	.	.	.	.	.	.	.	.	..	

fore, thought to be adequate for the present purpose, but the frequency of failure of 1:1 segregation will not be considered. The occasional irregular asci have been placed in the classes they most nearly resemble.

In Table 2 the asci, designated by the classes shown in Table 1, are listed in the order in which they were isolated. Those which could not be scored are designated by NS.

Twin ascus phenotypes: Admittedly the method employed to detect twin phenotypes is a crude one. Observation of immature asci at magnifications high enough to allow detection of twin morphologies has shown that when the ascus

TABLE 2

The order in which asci were isolated from each perithecium. The ascus class designations used are those shown in Table 1. NS = not scored

P-1	P-3 cont.	P-4	P-4 cont.	P-5 cont.	P-6 cont.	P-7	P-7 cont.	P-8 cont.	P-9 cont.
2T-3-4	NS	2NS	PD-1-2	2PD-1-1	PD-1-1	PD-1-1	PD-1-1	3PD-1-1	PD-1-1
PD-2-2	PD-1-2	T-1-7	T-1-6	PD-2-3	PD-2-3	T-1-3	T-1-2	T-1-2	PD-1-2
PD-2-4	PD-1-1	T-1-3	T-3-2	T-1-4	PD-1-1	2PD-1-1	PD-1-2	T-1-4	T-1-3
2PD-1-1	PD-1-2	T-1-1	2PD-2-1	PD-1-1	PD-1-2	PD-2-1	PD-1-1	PD-1-1	T-1-7
2PD-2-3	PD-2-2	PD-1-1		NS	NS	T-3-3	T-1-4	T-1-6	2PD-1-1
PD-1-1	NS	NS	P-5	PD-2-2	T-1-2	PD-2-4		3PD-1-1	T-1-8
	2T-1-1	2PD-2-3	T-2-5	PD-1-2	PD-2-2	NS	P-8	PD-2-2	T-2-7
P-2	PD-1-1	2PD-1-2	PD-2-4	PD-1-1	T-1-3	T-1-1	PD-1-1	PD-1-1	PD-2-3
3PD-1-1	2T-1-1	PD-1-1	PD-1-2	PD-1-2	PD-1-2	3PD-1-2	PD-1-2	PD-2-1	PD-1-1
PD-1-2	PD-1-2	T-1-7	NS	NS	T-1-4	NS	T-3-3	T-1-2	T-1-1
PD-2-1	NS	PD-1-2	PD-1-2	2PD-1-2	PD-1-1	PD-1-1	T-1-8	PD-2-1	PD-1-2
PD-1-1	2PD-1-1	T-1-2	PD-2-2	2T-1-7	T-1-7	PD-1-2	T-2-5	PD-2-2	2PD-1-1
PD-1-2	2PD-1-2	PD-1-1	2PD-1-1	PD-1-2	PD-2-1	T-1-6	PD-1-1		PD-2-3
PD-1-1	PD-1-1	PD-2-4	PD-2-2	T-1-5	PD-1-2	PD-1-2	PD-1-2	P-9	T-2-5
2PD-1-2	T-1-6	PD-1-1	T-1-8	NS	T-1-5	2PD-1-1	PD-1-1	PD-2-2	T-1-7
2PD-1-1	T-1-2	PD-2-4	PD-2-4	PD-1-2	PD-1-2	T-1-7	PD-1-2	PD-1-1	PD-2-4
PD-1-2	T-1-6	PD-2-2	2PD-1-2	PD-2-3	T-1-6	T-1-8	T-3-6	2PD-1-2	NS
PD-1-1	PD-1-2	PD-1-1	PD-1-1	PD-1-1	PD-1-1	4PD-1-2	PD-1-2	T-1-7	PD-2-2
2PD-1-2	PD-2-3	2PD-1-2	NS	NS	PD-1-2	PD-2-2	PD-2-4	PD-1-2	PD-1-1
	T-1-7	NS	T-1-6	T-1-2	PD-1-1	PD-1-2	PD-1-1	T-1-2	PD-2-4
P-3	2NS	PD-2-1	T-1-5	PD-1-2	2PD-1-2	PD-1-1	T-1-8	6PD-1-2	PD-2-1
T-1-8	PD-1-2	PD-1-1	PD-1-2		2PD-2-1	PD-1-2	PD-1-2	PD-2-3	3PD-1-2
PD-1-2	PD-2-1	PD-1-2	PD-1-1	P-6	PD-2-4	T-1-5	T-2-5	2PD-1-1	PD-1-1
NS		2PD-1-1	NS	PD-1-1	PD-2-1	PD-2-2	PD-2-3	T-1-8	PD-2-1
T-1-8		NS	T-3-3	PD-1-2	T-1-8	PD-1-2	PD-1-2	NS	
PD-1-2		PD-1-1	T-1-5	PD-1-1	PD-2-4	PD-1-1	PD-2-3	T-1-7	
3NS		T-1-6	NS	PD-1-2	T-3-7	T-1-2	PD-1-2	PD-1-1	
3PD-1-1		T-1-7	PD-1-2	PD-2-2		T-2-5	NS	T-1-5	

cluster is placed on a flat surface the members of a twin pair may, perhaps more often than not, be separated by several other asci and be adjacent only at their attached ends. However, the coincidences, shown in Table 2, of the same T-1 or PD-2 subclass are thought to indicate twinning. In these two main classes of intermediate frequency there are 30 cases in which more than one ascus of a subclass was obtained from the same perithecium and in 21 of these cases two of the same subclass were isolated consecutively or were separated by one, two, three or four asci of another type. Also, if the two T-3-4 asci are not twins then their consecutive isolation from the same perithecium would appear to represent an unlikely event.

This indication of adjacently located twin phenotypes, coupled with the frequent observation of degenerate twins, both showing the same symptoms of degeneracy, leads to the conclusion that the phenotypes of twins must be determined simultaneously for both members by the same events. Such a conclusion

conflicts, of course, with current hypotheses which attribute the determination of ascus phenotype to segregations occurring at meioses assumed to take place in individual asci which, prior to meiosis, represent zygotes, each consisting of a single cell with a single diploid nucleus. If this were the situation, the segregation pattern of any ascus would be independent of that of its neighbors in the ascus cluster. The regular observation of twin asci seems, therefore, to be irreconcilable with the hypothesis that meiosis occurs in preformed asci.

Other observations concerning the structure of immature asci also make this hypothesis appear unlikely (MITCHELL 1960). When seen with phase contrast, the ascus content appears to consist of several hyphae which have associated intimately with one another to form the spores and their matrices. Their proximity during development suggests that a twin pair may be formed by the same group of hyphae, the twin phenotypes being determined by the genetic constitutions of these hyphae.

Spore pair order: Although indications of non randomness have been reported (CATCHESIDE 1944; WHITEHOUSE and HALDANE 1945) the various subclasses within a main ascus class, for example PD-1-1 and PD-1-2, have usually been assumed to be equally probable, reflecting merely the various possible positions of the four nuclei following meiosis in the ascus. However, from the results with respect to the tetratype-2 class it seems rather unlikely that, in this cross, the eight T-2 subclasses were equally frequent. If this were so, then the probability of getting the result observed, that is, five of the six T-2 asci in one subclass and one in another would be 1/6250. A hitherto unrecognized significance of spore pair order seems, rather, to be suggested.

The observation that four of the T-2 asci were isolated consecutively or second consecutively with T-1-8 or T-1-7 has given rise to the speculation that the T-2 and T-3 asci do not constitute separate segregation classes but, rather, spore-pair rearrangements of T-1. This speculation has not led to a simple explanation of the improbable distribution of the T-2 subclasses since pursuit of it in this direction seems to require that significance be attached to the suggested deficiency of T-1-3 and T-1-4 asci. However, it also seems necessary to assume that a particular type of spore-pair rearrangement is more likely and this possibility is regarded as interesting because it might reflect unknown details of ascus construction. Nonlinear asci are not uncommon, particularly in some crosses. Such asci can be observed to attain linearity through rearrangement and the relationship of the spores to their matrices may be such as to favor pair-wise rearrangement. The possibility that the T-2 and T-3 asci arose by rearrangement is, of course, also interesting in connection with the conventional interpretation of them as segregation patterns produced by double crossovers.

Ratios of ascus classes: The relative frequencies of the ascus classes shown in Table 1, parental ditypes 1 and 2, tetratypes 1, 2 and 3 (BARRATT, NEWMEYER, PERKINS, and GARNJOBST 1954) have formerly been interpreted as reflecting the frequencies of crossing over between the loci of the two markers, if they show linkage, and between these loci and the centromere of their chromosome. Such an interpretation of the data presented would place *cot* about 24 map units from the

centromere with *pyr* almost equidistant both from the centromere and from *cot*. However, the ratio of parental ditypes to tetratypes, 209:73, is very nearly 3:1 while the PD-1 to PD-2 ratio, 157:52, is almost precisely 3:1. This result is viewed with considerable interest because the sample is larger and was more uniformly classifiable than average and because ratios suggested by other data have already led to the suspicion that such a result may be indicative of something other than a very tidy spacing of genes along the chromosome (MITCHELL 1960).

The possibility in mind is that the apparent linkage is due, not to physical linkage between the loci of two single-factor characters but rather, to the presence of a larger number of independently segregating factors which affect expression of the phenotypes scored. It may be seen that the frequency of wild segregants from the asci tabulated was very nearly 6.25 percent, the expected frequency of wilds from a cross involving four unlinked markers, if meiosis of a fusion nucleus precedes ascospore formation. It is also the expected frequency, in the absence of linkage, of the double recessive class in the F_2 of a dihybrid cross in diploid organisms. Other observations commonly made also suggest involvement of a larger number of factors. Often, within a phenotype class there may be seen differences in the degree of expression such as to suggest that the phenotype is arrived at in several different ways. Also, as might be expected if this is true, recombinant frequencies from crosses involving different isolates of the same markers often vary, and there are indications that the variation is discontinuous (MITCHELL 1958). Crosses of segregants from the asci described here are being studied in order to learn more about shifts in ascus class ratios.

Inheritance of perithecial characters: It is commonly observed that perithecia from the same cross vary with respect to such details as size, thickness of walls, morphology of asci and spores, proportions of normal and degenerate asci, and viability of spores. Observations made in connection with the cross described here indicate that at least some of these traits are heritable, although not in an obviously straightforward way. As mentioned above, the parents were obtained from the only highly fertile perithecium among about 30 from the first cross. Also, germination of spores from the exceptional perithecium was much more nearly complete than that of spores from a few of the less fertile ones. In the daughter cross the exceptional fertility and spore viability remained, but differences between perithecia with respect to ascus morphology were seen. Intra-ascus crosses of segregants from perithecia 8 and 9, which showed different ascus morphologies, have now been made and, in each case, characteristics associated with the parent perithecium were again observed. This may be useful since it suggests the possibility of selecting, for a given cross, desired attributes of perithecium and ascus morphology. It is also interesting because it suggests segregations of genetic factors prior to perithecium formation. This recalls characteristics of vegetative growth of certain strains discussed earlier (MITCHELL 1959). These characteristics include regularly occurring and heritable shifts in phenotypic expression and general occurrence of sectoring in colonies from single conidia or ascospores.

In data from other crosses from which individual perithecia were analyzed

indications have been seen that the perithecia of one cross may differ with respect to the relative frequencies of ascus classes. In the data given here there is little to suggest such differences. Perithecium 2 may have been exceptional but the sample is too small to allow a decision. However, it seems possible that in crosses between less closely related strains the occurrence of differences of this sort may tend to blur both progeny and ascus class ratios.

SUMMARY

Asci from an exceptionally fertile cross were analyzed in a way which is thought to indicate strongly that pairs of asci formed adjacently in the perithecium may be regularly identical with respect to patterns of segregation of known markers. This result indicates a need for revision of current hypotheses attributing the determination of ascus phenotypes to segregations occurring at meiosis in preformed asci.

The occurrence of certain ascus classes in almost precise 3:1 ratios suggests, along with other observations briefly discussed, that recombinant frequencies of less than 50 percent from two-marker crosses may not necessarily indicate physical linkage. Even though the progeny can be placed in the expected four main phenotypic classes, the possibility of several independently segregating factors having related and interacting effects on phenotypic expression seems an interesting one to consider.

The observation of a rather striking preference for one particular spore pair order among eight supposedly possible ones suggests that the spore pair order may have significance with which it is not ordinarily credited. The more probable orders within twin ascus pairs appear to be identical or exactly reversed. This is thought, perhaps, to reflect some detail in the mechanism of ascus formation but to be complicated by spore pair rearrangements within the ascus.

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MODIFICATION OF ABNORMAL GAMETIC RATIOS IN DROSOPHILA. I. EVIDENCE FOR AN INFLUENCE OF Y CHROMOSOMES AND MAJOR AUTOSOMES ON GAMETIC RATIOS FROM BAR-STONE TRANSLOCATION MALES[1]

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IF one considers the meiotic behavior of two pairs of non-homologous chromosomes, designating one pair A and A′ and the other pair B and B′, then it is expected that A will separate from A′ and B from B′ and that, following a random assortment of the two pairs of chromosomes, four gametic types (AB, A′B′, AB′ and A′B) will be produced with, under ordinary circumstances, equal frequency.

NOVITSKI and I. SANDLER (1957) reported a case in *Drosophila melanogaster* in which they showed that, despite the absence of evidence for departures from regular separation and random assortment in their experiments, certain gametic types were, nonetheless, recovered appreciably more frequently than others.

They studied the kinds of gametes produced by Drosophila males carrying the Bar translocation of STONE, T(1;4)B^{S}, where A or X^{D} represents the large distal piece of a broken X chromosome attached to the centric piece of a broken fourth chromosome, and A′ or IV a normal fourth chromosome, and where B or X^{P} represents the proximal centric piece of the broken X chromosome, marked with the mutant Bar, and having the remaining acentric piece of the broken fourth chromosome attached to its free end, and B′ or Y a normal Y chromosome (Figure 1). The relative frequencies of the four gametic types, $X^{D} + X^{P}$, IV + Y, X^{D} + Y, and IV + X^{P}, observed in their experiments are given below in row I, and the relative rates of recovery of the members of each homologous pair of chromosomes are given in row III.

	$X^{D} + X^{P}$	IV + Y	X^{D} + Y	IV + X^{P}
I.	26.5	19.1	11.6	42.8
II.	26.4	19.0	11.7	42.9
	X^{D}	IV	X^{P}	Y
III.	38.1	61.9	69.3	30.7

For each gametic type, the expected frequency was calculated by multiplying the over-all frequency of recovery of one of the components by the over-all frequency of recovery of the other. The expected values are given in row II and were obtained by multiplying the appropriate values in row III. The fact that

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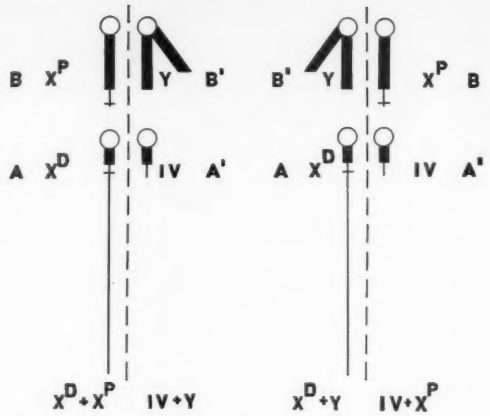

FIGURE 1.—The two kinds of orientation of the paired homologues that give rise to the four gametic types, $X^D + X^P$, IV + Y, X^D + Y and IV + X^P. (Adapted from NOVITSKI and SANDLER 1957).

the observed and expected frequencies are in agreement argues for the independence of X^D–IV and X^P–Y segregations despite distortion in the individual segregation ratios.

The egg count experiments by NOVITSKI and SANDLER (1957) gave no evidence of differential zygote mortality; from general considerations, they thought it unlikely that gametic lethality, as commonly conceived, or sperm competition could account for the observed abnormal gametic ratios. They suggested as a tentative explanation that not all products of spermatogenesis are functional and that certain components of the translocation—the longer ones (X^D and Y)—were segregating preferentially into the nonfunctional meiotic products.

The preliminary experiments to be discussed in the present paper will present evidence (1) that results such as those described above are subject to considerable variation in that, under certain conditions, the distorted ratios essentially disappear, and (2) that otherwise ordinary Y chromosomes and major autosomes, derived from different common laboratory stocks, appear to be responsible for the variation, for in the presence of *E* ("equal ratios") Y autosome combinations, aberrant ratios tend to disappear, while in the presence of *A* ("abnormal ratios") Y autosome combinations, striking inequalities are observed. A brief report of this work was made elsewhere (ZIMMERING 1959).

Experiments demonstrating the difference in behavior of B^s *males after outcrosses to attached-X females from different common laboratory stocks:* In order to recover the four different kinds of sperm produced by males carrying the B^s translocation, such males are crossed to attached-X females carrying a Y chromosome ($\overline{XX}$/Y), and to those carrying X^P in place of the Y chromosome ($\overline{XX}/X^P$). It may be seen from an examination of Table 1 that the gametic types IV + Y and IV + X^P are recoverable from both crosses, while the gametic type $X^D + X^P$

is recoverable only from the former cross, and the gametic type $X^D + Y$ only from the latter. The figures given in parentheses in Table 1 represent estimates of the number of $X^D + Y$ sperm expected from the former cross and the number of $X^D + X^P$ sperm expected from the latter. The estimates are based simply on the assumption that in both kinds of matings, $X^D + X^P$ and $IV + Y$ sperm appear in the same ratios to each other as do $X^D + Y$ and $IV + X^P$ sperm.

A surprising result was obtained in one of the first experiments carried out, where a single B^S translocation male was mated to an $\overline{XX}/Y$ female homozygous for the X chromosome marker *y* (yellow body), and the F_1 B^S males were tested by crosses with their $\overline{XX}/Y$ and $\overline{XX}/X^P$ sisters. These F_2 results are given in Table 1. It seems clear on inspection that, while the expected frequencies of the four gametic types calculated on the assumption of random assortment are in very good agreement with those observed, the wide departures from equality among the gametic types and between members of homologous pairs of chromosomes found by NOVITSKI and SANDLER in their experiments are clearly absent. It should be pointed out, also, that a chi-square test of the difference between the observed and expected numbers in each of the gametic classes, calculated on the assumption that the classes occur equally frequently, indicated no significant difference.

Additional tests were therefore carried out to determine if the behavior of the translocation components of the B^S male was modifiable such as to give results more in line with those found by NOVITSKI and SANDLER. For this purpose, B^S males, taken from the same stock used in the previous experiment, were crossed to attached-X females from two other common laboratory stocks, and the F_1 B^S

TABLE 1

Progeny of single pair matings of F_1 B^S translocation males by F_1 attached-X sisters homozygous for y and carrying a Y chromosome or the proximal fragment, X^P, of the B^S translocation. Both males and females were derived from a single pair P_1 mating of a B^S translocation male with an attached-X female homozygous for y*

Constitution of eggs		Constitution of sperm $X^D + X^P$	$IV + Y$	$X^D + Y$	$IV + X^P$
XX		lethal	628	lethal (647)	657
Y		701	lethal	lethal	lethal
XX		lethal (582)	521	lethal	583
X^P		semi-lethal	lethal	574	lethal
	n	1283	1149	1221	1240
	obs.	26.2	23.5	25.0	25.3
	exp.	26.4	23.7	24.8	25.1

Over-all frequency of each translocation component

X^D	IV	X^P	Y
51.2	48.8	51.5	48.5

* *y*=yellow body.

males tested by crosses with their $\overline{XX}/Y$ and $\overline{XX}/X^P$ sisters. In one series of experiments, three single pair P_1 matings were made of B^S males and $\overline{XX}/Y$ females homozygous for the X chromosome markers *y*, *v*, and *car*, and in the other, three single pair P_1 matings were made of B^S males and $\overline{XX}/Y$ females homozygous for the X chromosome markers *y*, *w*, and *bb*. The F_1 males were mated with their $\overline{XX}/Y$ and $\overline{XX}/X^P$ sisters. The F_2 results from each of the three P_1 matings in the former series and from each of the three P_1 matings in the latter are give in Tables 2 and 3, respectively. The results in these tables are given in a form somewhat different from that shown in Table 1. In Tables 2 and 3, the first and second rows give the number of offspring, both recovered and calculated, in each of the classes from matings of F_1 males by $\overline{XX}/Y$ and $\overline{XX}/X^P$ sisters, respectively. The data in Table 2 do not suggest, on the whole, gross inequalities in the recovery rates of the four types of sperm or between X^D and IV, and X^P and Y. However, a chi-square test of the difference between the observed and expected numbers in each gametic class, gave P values of much less than .01. Deviations from randomness as large as, or larger than, these were also found in experiments yet to be discussed, and a comment about these will be deferred until the results from all experiments have been discussed.

On the other hand, an effect resembling that found by NOVITSKI and SANDLER is indicated by the results given in Table 3. Here, while the over-all effect is not

TABLE 2

Progeny of single pair matings of F_1 B^S translocation males by F_1 attached-X sisters homozygous for y v* car† *and carrying a Y chromosome or X^P (See text for details)*

		Constitution of sperm			
		$X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P
I.		252	312	(294)	324
		(428)	530	577	636
	n=	680	842	871	960
	obs.	20.3	25.1	26.0	28.6
	exp.	22.6	27.4	23.6	26.3
II.		342	332	(512)	425
		(360)	349	417	346
	n=	702	681	929	771
	obs.	22.8	22.1	30.1	25.0
	exp.	25.3	24.6	27.6	22.5
III.		742	752	(1011)	758
		(383)	388	439	329
	n=	1125	1140	1450	1087
	obs.	23.4	23.7	30.2	22.6
	exp.	24.7	25.0	28.9	21.3

Over-all frequency of each translocation component

	X^D	IV	X^P	Y
I.	46.2	53.7	48.9	51.0
II.	52.9	47.1	47.8	52.2
III.	53.6	46.3	46.0	53.9

* *v*=vermilion eye color.
† *car*=carnation eye color.

TABLE 3

Progeny of single pair matings of F_1 B^S translocation males by F_1 attached-X sisters homozygous for y w* bb† *and carrying a Y chromosome or X^P (See text for details)*

		Constitution of sperm			
		$X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P
I.		235	195	(122)	339
		(369)	306	208	579
	n=	604	501	330	918
	obs.	25.7	21.3	14.0	39.0
	exp.	25.7	21.3	14.0	39.0
II.		75	83	(50)	118
		(40)	45	32	76
	n=	115	128	82	194
	obs.	22.2	24.7	15.8	37.4
	exp.	22.6	25.2	15.4	37.0
III.		24	29	(22)	46
		(215)	260	165	353
	n=	239	289	187	399
	obs.	21.5	25.9	16.8	35.8
	exp.	21.9	26.3	16.4	35.4

Over-all frequency of each translocation component

	X^D	IV	X^P	Y
I.	39.7	60.3	64.7	35.3
II.	38.0	62.1	59.6	40.5
III.	38.3	61.7	57.3	42.7

* *w* = white eye.
† *bb* = bobbed bristles.

quite as striking as that found by them, (1) sperm carrying $X^D + Y$ are recovered appreciably less frequently than those carrying IV + X^P, (2) the recovery rates of the members of homologous pairs of chromosomes show marked departures from equality, and (3) very good agreement is found between the observed and expected frequencies of the four gametic types.

Evidence that the abnormal gametic ratios are probably not related to events occurring in the F_1 female: It has been shown, in crosses involving B^S males by attached-X females homozygous for *y w bb*, that the F_2 offspring give evidence of an unequal recovery of gametic types, especially between the complementary classes, $X^D + Y$ and IV + X^P. It will be recalled that from crosses of B^S males to any $\overline{XX}/X^P$ females a sperm carrying $X^D + Y$ is recoverable when it fertilizes an egg containing X^P, and a sperm carrying IV + X^P is recoverable when it fertilizes an egg containing the attached-X chromosome. It is assumed that these complementary classes of eggs are produced with equal frequency; consequently, the aberrant ratios observed are interpreted as being the result of events which occur in the F_1 B^S male. If for some reason, however, functional eggs carrying X^P are produced less frequently than those carrying the attached-X chromosome, this inequality would result in a deficiency in the recovery of sperm carrying $X^D + Y$. There is no reliable check on this point since the class that might give the necessary information is the hyperploid male class which has a very low and

erratic viability in all experiments. The following tests were carried out in an attempt to decide between these alternatives. Males from the original B^s stock were single pair mated with females selected from each of three common laboratory stocks. Each of these females carried the so-called "double X" chromosome (reversed acrocentric X) homozygous for the X chromosome markers *y* and *f* (forked bristles). The F_1 males were mated, not to their sisters, but to *y*, $\overline{XX}/Y$ and *y*, $\overline{XX}/X^P$ females taken at random from a stock which had been made up by simply selecting F_2 males and females from the experiment, the results of which were given in Table 1. It will be recalled that these results gave no evidence of departures from equality of recovery of the four gametic types. The results in Table 4 show that the first two experiments in this series give evidence of marked distortions in gametic ratios. There is no obvious reason to believe that a change had occurred in the "tester female" stock such that, in these two crosses, the $\overline{XX}/X^P$ females from the tester stock resembled the F_1 *y w bb*, $\overline{XX}/X^P$ females; then it would have to be supposed that a non random selection of these tester females was made such that those that had *not* undergone this change were used in the last experiment of this series, in which the results indicate a vastly weaker effect. The results may, therefore, be taken as indicating no reason to assume any contribution to this effect on the part of the $\overline{XX}/X^P$ female employed in the various experiments described. It might be worth mentioning that these results also show that this phenomenon is not related to the specific X chromosome

TABLE 4

Progeny of the mating of F_1 h10 males, derived from crosses with "double X" females from each of four different stocks, with attached-X females homozygous for y *and carrying a Y chromosome or X^P*

		Constitution of sperm			
		$X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P
I.		709	652	(445)	1158
		(520)	478	338	879
	n=	1229	1130	783	2037
	obs.	23.7	21.8	15.1	39.3
	exp.	24.4	22.5	14.3	38.5
II.		491	454	(344)	992
		(323)	299	193	556
	n=	814	753	537	1548
	obs.	22.3	20.6	14.7	42.4
	exp.	23.9	22.2	13.1	40.8
III.		897	932	(980)	1152
		(743)	772	744	875
	n=	1640	1704	1724	2027
	obs.	23.1	24.0	24.3	28.6
	exp.	24.5	25.4	22.9	27.2

Over-all frequency of each translocation component

	X^D	IV	X^P	Y
I.	38.8	61.1	63.0	36.9
II.	37.0	63.0	64.7	35.3
III.	47.4	52.6	51.7	48.3

markers carried by the P_1 females, since in all three runs, the P_1 females were phenotypically *y* and *f*. Significant deviations from random assortment ($P = 0.01$) were found in the second and third experiments of this series, but no significant deviation was found in the first ($P = 0.20$–0.05).

Tests of another X-4 translocation male: It was of interest to determine if such abnormal gametic ratios would be found in corresponding classes from another X-4 translocation, unrelated in origin to the B^s translocation but having a break in the X chromosome at a position similar to that of the B^s translocation. A number of X-4 translocations were assayed, and one, T(1;4)h3, was selected for further testing. By means of simple experiments, it was possible to determine the approximate position of the break in the X chromosome in the h3 stock. Males carrying the h3 translocation were crossed with attached-X females carrying X^p of the B^s translocation, and B^s translocation males were crossed with attached-X females carrying X^p of the h3 translocation. The first cross gave fully viable and fertile (as proved by later tests) males showing the B^s phenotype and a few hyperploid males with the B^s phenotype. From the second cross, a few hyperploid males showing the B^s phenotype were recovered, but no non-B^s males were found. The absence of the non-B^s male class from the second cross probably means that males carrying X^D from the B^s translocation and X^p from the h3 translocation do not survive presumably because X^p of the h3 translocation is shorter than X^p of the B^s translocation, with the result that the combination of X^D from the B^s translocation and X^p from the h3 translocation gives rise to a deficient X chromosome which does not permit males carrying such an X chromosome to survive. An examination of the female offspring from a cross of h3 males and attached-X females homozygous for the X chromosome markers, *y v car*, showed that females receiving X^p from the h3 male were phenotypically *y* and *v*, but not *car*. It appears, then, that the break in the X chromosome in the h3 translocation is somewhere between the break position of the B^s translocation and *car*. X^p of the h3 translocation is, therefore, a bit shorter than X^p of the B^s translocation. The F_2 results of matings of h3 translocation males with *y*, $\overline{XX}$/Y and *y w bb*, $\overline{XX}$/Y females are given in Table 5. It should be noted that these females were derived from the same stocks employed earlier in the crosses with B^s males (see Tables 1 and 3), and that the F_1 males from each cross were mated with attached-X females from a common tester stock, namely, *y v car*, $\overline{XX}$/Y and *y v car*, $\overline{XX}/X^p$. It seems reasonably clear from these results that h3 translocation males are behaving in a manner not unlike that of the B^s translocation males in that (1) both show considerable variation, and (2) both show strong effects as F_1 offspring from outcrosses to the *y w bb*, $\overline{XX}$/Y females, and a very much weaker effect after outcrossing to *y*, $\overline{XX}$/Y females. It may be taken, therefore, that the unusual behavior of males carrying the B^s translocation is not related specifically to the B^s translocation *per se* but may be related to some property common to males carrying X-4 translocations of this general type. No significant deviation from random assortment was found in the second experiment ($P = 0.20$–0.05) and a deviation on the borderline of significance was found in the first ($P = 0.05$–0.01).

TABLE 5

Progeny of single pair matings of F_1 h3 translocation males and attached-X females homozygous for y v car *and carrying a Y chromosome or X^P of the h3 translocation. The F_1 males were derived from a cross of an h3 translocation male by a female homozygous for* y w bb *and carrying a Y chromosome (I), and from a cross of an h3 translocation male by an attached-X female homozygous for* y *and carrying a Y chromosome (II) (See text for details)*

		Constitution of sperm			
		$X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P
I.		396	391	(262)	805
		(275)	272	170	523
	n=	671	663	432	1328
	obs.	21.7	21.4	14.0	42.9
	exp.	23.0	22.8	12.6	41.5
II.		728	736	(667)	764
		(703)	711	603	691
	n=	1431	1447	1270	1455
	obs.	25.5	25.8	22.7	26.0
	exp.	24.8	25.1	23.4	26.7

Over-all frequency of each translocation component

	X^D	IV	X^P	Y
I.	35.7	64.3	64.6	35.4
II.	48.2	51.8	51.5	48.5

Experiments to determine the relative effectiveness of Y chromosomes and autosomes of different origins in bringing about abnormal gametic ratios from B^S *males:* It has been established in the experiments thus far described that the anomalous ratios found in certain sets of F_2 data can be ascribed to events which occur in the F_1 B^S male. Furthermore, B^S males have been demonstrated to behave quite differently from each other as F_1 offspring from outcrosses to females from different common laboratory stocks. On a simple genetic basis, the difference in the behavior of the two kinds of males may be explained as the result of the presence of factors associated with one or more of the chromosomes contributed to the F_1 male by its parental attached-X female, namely, factors associated with the Y chromosome, the fourth chromosome, and the two major autosomes.

To test this idea, crosses were arranged such that the B^S males carried a varied mixture of chromosomes, derived from two different attached-X stocks. The difference between these stocks was that when the attached-X females from one of these were crossed to B^S males, clear evidence of abnormal gametic ratios was found in the F_2, and when the attached-X females from the other were used in the same manner, little or no evidence of distorted ratios was found in the F_2. Chromosomes derived from the former type of stock will be referred to as the *A* ("abnormal ratio") type, and those from the latter, as the *E* ("equality") type.

Males of six different genotypes were tested, and the tests were later repeated.

Other than X^D and X^P, which originated from a common stock, the Y chromosome and the autosomes were derived as follows:

	IV	Y	2	3
Type 1	*E*	*E*	*A/E*	*A/E*
Type 2	*A*	*E*	*A/E*	*A/E*
Type 3	*E*	*A*	*A/E*	*A/E*
Type 4	*A*	*A*	*A/E*	*A/E*
Type 5	*A*	*A*	*A/A*	*A/A*
Type 6	*E*	*E*	*E/E*	*E/E*

The following points about the genotypes of these males are worth noting. In Types 1–4, chromosomes 2 and 3 are each heterozygous for *A*- and *E*-derived chromosomes. However, in Types 1 and 2, the Y chromosome has come from the *E* stock, while in Types 3 and 4, the Y chromosome has been derived from the *A* stock. Furthermore, of the two types carrying Y-E, Type 1 carries a fourth chromosome of *E* origin; and Type 2, a fourth chromosome of *A* origin. Similarly, of the two types carrying Y-A, Type 3 carries a fourth chromosome derived from the *E* stock; and Type 4, a fourth chromosome which came from the *A* stock. Types 5 and 6 represent males carrying, respectively, all *A*-derived or all *E*-derived chromosomes. The results from the matings of these various males to $\overline{XX}$, *y v car*/Y and $\overline{XX}$ *y v car*/X^P females from a common tester stock are given in Table 6.

First, the effects of the fourth chromosome of different origins on the gametic ratios may be considered. Type 1 carries an *E*-derived fourth chromosome and Y chromosome, and its autosomes are heterozygous for *A*- and *E*-derived chromosomes, while Type 2 differs from it only in that it possesses an *A*-derived fourth chromosome. Inspection of the results shows that these males give sensibly similar gametic ratios. Type 3 possesses an *E*-derived fourth chromosome, and an *A*-derived Y chromosome, and is heterozygous for *A* and *E* in the major autosomes, while Type 4 differs from it only in the possession of an *A*-derived fourth chromosome. Clearly, the gametic ratios from Type 3 are no less abnormal than those from Type 4, yet the former has an *E*-derived, and the latter an *A*-derived fourth chromosome. Thus, under the conditions of the experiment, the data fail to give evidence for any importance of the origin of the fourth chromosome in the production of abnormal gametic ratios.

Some information regarding the relative effectiveness of an *A*-derived Y chromosome and of *A*-derived autosomes comes from the following experiments. Types 1 and 2 are heterozygous for *A*- and *E*-derived major autosomes and carry an *E*-derived Y chromosome, while Type 6 differs from these types in that the major autosomes are homozygous for *E*-derived chromosomes. A comparison of the results from these experiments shows that in Types 1 and 2 there is a small but consistent departure from the approximate equality among the four gametic types as found in Type 6. It appears, therefore, that *A*-derived autosomes have a dominant-like effect in themselves. The results from Types 3 and 4, which differ

TABLE 6

Progeny from the crosses of B^S *males of various* A *and* E *genotypes by a common tester attached-X female carrying a Y chromosome or* X^P *(See text for details)*

Male of type		Series 1 $X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P	Series 2 $X^D + X^P$	IV + Y	$X^D + Y$	IV + X^P
1		437	413	(474)	643	885	819	(819)	1064
		(210)	198	193	262	(800)	740	748	972
	n=	647	611	667	905	1685	1559	1567	2036
	obs.	22.9	21.6	23.6	31.9	24.6	22.8	22.9	29.7
	exp.	25.5	24.2	21.0	29.3	25.8	24.0	21.7	28.5
2		555	542	(612)	845	525	483	(463)	710
		(216)	211	216	298	(534)	491	481	738
	n=	771	753	828	1143	1059	974	944	1448
	obs.	22.1	21.5	23.7	32.7	23.9	22.0	21.3	32.7
	exp.	25.1	24.5	20.7	29.7	25.6	23.7	19.6	31.0
3		221	158	(128)	354	436	390	(236)	694
		(98)	70	54	149	(287)	257	180	529
	n=	319	228	182	503	723	647	416	1223
	obs.	25.9	18.5	14.8	40.8	24.0	21.5	13.8	40.6
	exp.	27.1	19.7	13.8	39.6	24.4	21.9	13.3	40.1
4		518	505	(405)	772	670	493	(423)	1052
		(389)	379	335	638	(355)	261	198	492
	n=	907	884	740	1410	1025	754	621	1544
	obs.	23.0	22.4	18.8	35.8	26.0	19.1	15.7	39.1
	exp.	24.6	24.0	17.2	34.2	27.2	20.3	14.5	37.9
5		323	272	(213)	797	460	327	(210)	792
		(165)	139	86	321	(648)	461	293	1102
	n=	488	411	299	1118	1108	788	503	1894
	obs.	21.1	17.7	12.9	48.3	25.8	18.3	11.7	44.1
	exp.	23.6	20.2	10.4	45.8	26.2	18.8	11.3	43.7
6		1025	1036	(1280)	1112	2361	2432	(2873)	2619
		(279)	282	320	278	(1504)	1549	1753	1589
	n=	1304	1318	1600	1390	3865	3981	4626	4208
	obs.	23.2	23.5	28.5	24.8	23.2	23.9	27.7	25.2
	exp.	24.8	25.1	26.9	23.2	24.6	25.3	26.3	23.8

from Types 1 and 2 only in that they carry an *A*-derived Y chromosome, give clear evidence of an exaggeration of the effect in that they show markedly abnormal gametic ratios. It would appear, then, that in the presence of autosomal heterozygosity for *A*- and *E*-derived chromosomes, the origin of the Y chromosome is a pivotal factor in bringing about the variation in gametic ratios. It should be pointed out that the *A*-derived Y chromosome and autosomes present in the Type 3 male came from its male parent, while comparable chromosomes in the Type 4 male came from its female parent. Since, in both cases, gametic frequencies showed departures from equality of similar degree, it would appear to be a matter of indifference as to which parent contributes these chromosomes. Whether or not both the Y chromosome and autosome(s) *must* enter from the same parent, in order for the effect to take place, remains to be determined. It would seem also that the greatest effect in the direction of inequality or equality of gametic

types is observed when the Y chromosome and all major autosomes are derived from the *A* stock (Type 5) or the *E* stock (Type 6), respectively, suggesting perhaps a dosage-like effect of the major autosomes.

Chi-square tests for random assortment gave the following results. In Series 1, five of the six runs gave P values of less than 0.01, while those from the remaining one (Type 3 male) showed no significant deviation (P = 0.5–0.2). In Series 2, the results from the runs of males of Types 1, 2 and 6 gave P values of less than 0.01; from those of Types 3 and 5, a P value of 0.8–0.5 each; and from those of Type 4, a P value of 0.05–0.01.

To illustrate in another way the kinds of results discussed above, the data below give the relative rates of recovery of X^D and IV, and X^P and Y for each of the experiments shown in Table 6.

Male of type	Series	X^D	IV	X^P	Y
1	1	46.5	53.5	54.8	45.2
	2	47.5	52.5	54.3	45.7
2	1	45.8	54.2	54.8	45.2
	2	45.2	54.8	56.6	43.4
3	1	40.7	59.3	66.7	33.3
	2	37.6	62.2	64.6	35.4
4	1	41.8	58.2	58.8	41.2
	2	41.7	58.3	65.1	34.9
5	1	34.0	66.0	69.4	30.6
	2	37.5	62.5	69.9	30.1
6	1	51.7	48.3	48.0	52.0
	2	50.9	49.1	49.1	50.9

One of the most striking features of these data, aside from the marked variation in recovery rate between homologous chromosomes, is that both pairs of homologues vary in the same direction in any one experiment. That is, the smallest departures from equality for both pairs are found in tests with the Type 6 male, and the largest deviations from equality for both in tests with the Type 5 male. Similar situations exist also for Types 1–4 which give intermediate values for both pairs of homologues. Now, the origin of the fourth chromosome is clearly not decisive in bringing about these variations (probably related to its relatively small size), while the origin of the Y chromosome and major autosomes are. One question that immediately arises is if the grossly anomalous behavior of the heteromorphic X^D-IV bivalent is dependent, in some way, upon the misbehavior of X^D-Y bivalent, or if, for example, given a homomorphic X^P-Y, in which the Y chromosome is *A*-derived and the autosomal-*A* requirement is met, the heteromorphic X^D-IV bivalent (or other contrived heteromorphic bivalents) will by virtue of the simple presence of such *A*-derived chromosomes exhibit grossly abnormal behavior. Experiments testing this possibility are at present underway. Of interest in this connection are the studies of segregation in maize in the presence of the abnormal chromosome 10. RHOADES (1942, 1952) and LONGLEY

(1945) found that the abnormal chromosome 10, when heterozygous with a normal 10, was not only preferentially included into the function megaspore, but was capable of causing preferential segregation in non-homologous heteromorphic bivalents, which in the absence of abnormal 10, showed normal segregation. Furthermore, in experiments testing simultaneously the segregation of two such heteromorphic bivalents, of which one was heterozygous abnormal 10, no deviations from random assortment could be demonstrated. Recently, RHOADES and DEMPSEY (1959) made the interesting finding that non-homologous heteromorphic bivalents will give distorted gametic ratios even in the presence of homozygous 10, that is, when abnormal chromosome 10 is present as a homomorphic bivalent. Since this phenomenon in maize depends upon the presence of structures not known, and upon the occurrence of events not normal to the Drosophila male, its interpretation, at least in these respects, cannot be extended to the present case.

While there is as yet no direct evidence regarding the necessity for the presence of *A*-derived autosomes in order for an *A*-derived Y chromosome to have its effect, in many of the experiments reported from which markedly abnormal gametic ratios were obtained, an examination of the data from individual pair matings gave evidence of heterogeneity, that is, while the vast majority gave clear evidence of abnormal ratios, some cultures showed a normal recovery of the gametic types. The distinction between the two types of cultures was very sharp. Such variation was found, for example, in experiments, the results from which are given in Table 6. Now, the Y-*A* chromosome in these experiments were descendant from a single ancestral Y chromosome while the autosomes were essentially a random mixture of chromosomes derived from an "*A*-type" stock, that is, unlike the case of the Y chromosome, individual autosomes were not tested to determine if they were of the *A* or *E* type. It might be supposed, therefore, that in those cultures giving a normal recovery of gametic types, a necessary *A*-autosome component was absent (or not operating). Of course, other possible factors such as local environmental conditions or some other unsuspected genetic difference cannot be ruled out at present.

The same general reasoning, that is, mixtures of autosomal types, etc., may provide an explanation for the observed culture-to-culture heterogeneity found in some experiments whose over-all results indicated a more normal recovery of the translocation components. In any event, such heterogeneity could account, at least for the most part, for the significant deviations from randomness found in many of these experiments.

Some information has been obtained on the effects of certain autosomal inversions on the non random recovery of gametic types. A comparison was made of gametic ratios from tests of B^s males of the following Y autosome constitutions:

Expt. "E"–Y-*E*; *Cy/E*; *Sb/E*
Expt. "A"–Y-*A*; *Cy/A*; *Sb/A*

with dominant Curly (*Cy*) marking inversions (Ins (2L+2R)) and Stubble (*Sb*) marking the Moire inversion (In(3R)Mo). Given in terms of the relative rates

of recovery of X^D and IV, and X^P and Y, irrespective of their autosomal constitutions, the results were:

	X^D	IV	X^P	Y
Expt. "E"	50.0	50.0	48.7	51.3
Expt. "A"	40.5	59.5	64.8	35.2

It appears, then, that the combination of the *Cy* and *Sb* inversions has no demonstrable modifying influence, giving, as it were, essentially an *E*-type effect. This appears to rule out the possibility that, at least, when both autosomes are heterozygous for inversions, this condition is in itself sufficient to give an *A* effect. The abnormal gametic ratios found in the latter experiment may be regarded, therefore, as simply being the result of the presence, and evidently undiminished action, of the *A*-derived chromosomes. Disregarding those cultures which did not show a normal recovery of the translocation components in experiment "E", and those not giving abnormal ratios in "*A*", the frequencies of the various autosomal types among the gametes carrying each of the four translocation components turns out to be as follows:

		Cy Sb	++	*Cy* +	+ *Sb*	*No. of flies scored*
"*E*"	X^D	25.7	24.2	25.9	24.2	3578
	IV	25.0	25.0	26.1	23.9	
	X^P	23.7	24.9	26.6	24.8	
	Y	26.9	24.7	24.2	24.2	
"*A*"	X^D	24.7	27.9	23.7	23.7	5619
	IV	23.4	26.7	26.6	23.3	
	X^P	23.6	26.5	26.1	23.8	
	Y	23.9	28.1	25.4	22.6	

Examination of these data do not suggest any appreciable difference between the results from the two experiments, and gives no evidence for any wide departures from the expected random assortment of the autosomes into the four types of gametes. Essentially, then, under the conditions of the experiment, it may be concluded that the simple inclusion of an *A*-derived autosome, or its inversion-bearing homologue into a secondary spermatocyte is not sufficient to impede the cell containing one or the other of these chromosomes in its ultimate participation in fertilization.

Since the heterochromatic Y chromosome and the major autosomes from different common laboratory stocks have been demonstrated to show considerable variation with regard to their effect in producing abnormal gametic ratios, and since it would seem a distinct possibility that the heterochromatic regions of the autosomes are principally involved, variations were also looked for in the heterochromatic bases of different X chromosomes used in these experiments. No evidence for such variation was found. Bases from presumably three different X chromosomes tested in these experiments, namely, that marked with B^s *car*$^+$, or B^s *car* (derived by crossing over of a B^s *car*$^+$ chromosome with an X chromosome

carrying *car*, and hence carrying the base of the latter), or of the h3 translocation, gave substantially the same results. However, a wider variety of X chromosomes will have to be analyzed before such a possibility can be dismissed.

Other cases of distorted gametic ratios in the Drosophila male have been reported which have been shown to be subject to considerable modification depending upon the genetic factors associated with the bivalent showing distortion and upon those associated with non-homologous chromosomes. These cases include those (1) exhibiting various ways in which certain aspects of the segregation-distortion phenomenon may be modified according to the composition and origin of the homologue of the *SD*-bearing chromosome, and on the X chromosome (SANDLER, HIRAIZUMI and SANDLER 1959; SANDLER and HIRAIZUMI 1959); (2) suggesting a dependence of the non random recovery of the attached-XY chromosome and one of a number of heterochromatic fragments of the X chromosome upon the particular fragment used and the autosomal constitution (LINDSLEY and SANDLER 1958); (3) showing, not unlike the B^s case, the influence of Y chromosomes and autosomes on the non random recovery of certain X chromosomes recovered from irradiated populations (NOVITSKI and HANKS 1959). It should be mentioned that NOVITSKI (1947) presented some evidence suggesting that the "male sex ratio" phenomenon in *D. affinis* may also be subject to an autosomal modifying influence.

Aside from the marked differences in the behavior of the B^s translocation which result when "modifying" genes are present in non-homologous chromosomes, and when, apparently, the dosage of these "modifying" genes is changed, there is evidence (not surprisingly), also, for a modifying effect of temperature. Thus, for example, B^s males of the *A* type, when raised at 18°C and tested at 26°C, give no evidence of abnormal gametic ratios, while their brothers, raised and tested at 26°C, give gametic ratios showing the typical wide departures from equality. These experiments will be reported on in detail in a later publication. It should be pointed out that NOVITSKI and HANKS (unpublished) have demonstrated an effect of both low and high temperatures in their material.

The question of whether some products of spermatogenesis may be regularly nonfunctional cannot be answered by the data presented in this paper, since it may be argued that, in cases where normal gametic ratios are found, each translocation component has the same probability of inclusion into a nonfunctional (or functional) meiotic product. The possibility that the nonfunctioning of certain sperm types is associated with some peculiarity of the particular combination of chromosomes involved (a point raised by NOVITSKI and SANDLER) such that, perhaps, some oddity in the mechanics of meiosis results in the nonfunctioning of certain meiotic products, is to be explored.

Finally, some speculation might be made of the possible bearing of the results from these experiments on the commonly observed deficiency of XY gametes as compared with their complementary nullo X nullo Y gametes which arise from ordinary nondisjunction in the Drosophila male. This discrepancy has been interpreted as being the result of the loss of one of the two sex chromosomes from a potential XY spermatocyte, thus converting such a cell to one which will yield

either a regular X- or a regular Y-bearing sperm. Reasoning by analogy with the *A* effect in the B^s case, it is possible that at least a part of this discrepancy may be the result of the failure of a proportion of XY spermatocytes to proceed normally in the course towards fertilization.

SUMMARY

A genetic analysis was made of segregation of the B^s translocation in Drosophila males. Such males possess, as one pair of homologues, a fourth chromosome attached to a large distal piece of the X chromosome (X^D), and a normal fourth chromosome (IV), and as another pair, the proximal piece of the X chromosome (X^P) and the longer Y chromosome. It has been found that the relative frequencies of X^D and IV, and X^P and Y, and among the gametic types may show considerable variation. Results suggest that (1) otherwise indistinguishable Y chromosomes and major autosomes derived from different common laboratory stocks appear to be responsible for the variation, for in the presence of "*E*" (equality) Y autosome combinations, the inequalities tend to disappear, while in the presence of "*A*" (abnormal ratios) Y autosome combinations, distorted ratios are observed; (2) the greatest effect in the direction of equality or inequality is observed when the Y chromosome and all of the major autosomes are derived from the *E* or *A* stocks, respectively, intermediate effects being found in the presence of mixtures of *E*- and *A*-derived chromosomes; and (3) both pairs of homologues of the B^s male genotype always vary in the same direction in any one experiment, that is, where members of one pair of homologues show distorted ratios, so do members of the other, and where members of one pair are recovered with (more nearly) equal frequencies, so are members of the other.

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MEIOTIC DRIVE IN NATURAL POPULATIONS OF DROSOPHILA MELANOGASTER. IV. INSTABILITY AT THE SEGREGATION-DISTORTER LOCUS[1,2]

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IN a collection of flies from a natural population of *Drosophila melanogaster*, several second chromosomes have been isolated that contain, in the centromere region of chromosome II, a locus (named segregation-distorter and symbolized *SD*) that conditions, in heterozygous males, a highly abnormal segregation ratio in favor of the *SD*-bearing chromosome. Experiments bearing on the cytogenetic basis of the phenomenon of segregation-distortion and on the formal genetics of the *SD* locus have been reported by SANDLER, HIRAIZUMI, and SANDLER (1959) and by SANDLER and HIRAIZUMI (1959).

All of the *SD*-bearing chromosomes collected in nature, when tested in a "standard backcross" (i.e., *SD*/ *cn bw* ♂♂ × *cn bw* ♀♀), give very uniform segregation ratios. If we let k be the proportion of *SD*-bearing progeny in such a backcross, then the k value observed from any given male is almost always greater than 0.95. The *SD*-bearing chromosomes collected in nature, furthermore, all contain inversions in the right arm of the second chromosome which sharply reduce crossing over in that arm (crossing over in II L is approximately normal). Occasional recombinants, however, between the locus of *cn* and the locus of *bw* (cinnabar located at the base and brown at the tip of IIR) are recovered. Recombinants between *cn* and *bw* that contain the *SD* locus invariably behave differently from the original *SD*-bearing chromosomes in that the observed k values vary rather widely from test male to test male. These recombinants, furthermore, can be separated according to whether this variation in k is very wide—in which case the recombinant is referred to as *unstable*—or less wide—in which case the recombinant is referred to as *semistable*. The original *SD* lines are referred to as *stable*.

In this report it is proposed to consider the origin, the behavior, and the genetic basis of instability and semistability.

The origin of unstable and semistable SD *lines:* There are two different structural types among the original *SD* lines (SANDLER, HIRAIZUMI, and SANDLER 1959): a type represented by line *SD-5*, which carries two inversions in IIR and a lethal (which has thus far been inseparable from the more proximal of the inversions); and a type, represented by only one line, *SD-72*, which carries only the more distal of the inversions and no lethal. The *SD* locus itself is proximal to,

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and very closely linked to, *cn* (SANDLER and HIRAIZUMI 1959). In the case of *SD-72*, recombinants between *cn* and *bw* are recovered at a rate of about 0.25 percent (HIRAIZUMI, SANDLER, and CROW 1960). In the case of *SD-5*, the rate is exceedingly low except in one line (of the constitution *y f*: =/Y/+; *SD-5/ cn bw*) in which the rate is variable, but averages about 0.50 percent. The *y f*: = chromosome is a reversed acrocentric compound-X chromosome marked by the mutants, yellow and forked. Exactly why this particular line shows this high rate of exchange is not understood, but it is apparently a function of the X chromosomes since the high rate is maintained in spite of repeated backcrossing of *y f*: = /Y; *SD-5/ cn bw* females to *cn bw* males from the standard *cn bw* line. The inversion-bearing recombinants isolated from this line in free-X stocks, furthermore, do not exhibit this increased rate of exchange.

From *SD-72*, two types of recombinants between *cn* and *bw* are recovered: a *bw* recombinant carrying *SD* and lacking the *SD-72* inversion and, its complement, a *cn* recombinant carrying SD^+ and the inversion. Thus, for this case, it appears that the recombination takes place between the locus of *cn* and the proximal breakpoint of the inversion. From *SD-5*, four types of recombinants are recovered: a *bw* recombinant carrying *SD*, the lethal, and both inversions; and, its complement, a *cn* recombinant lacking the inversions and the *SD-5* lethal. Here, the recombination has apparently taken place between the distal breakpoint of the distal inversion and the locus of *bw*. Much more rarely, recombinants are recovered from *SD-5* heterozygotes that apparently result from an exchange between the locus of *cn* and the proximal breakpoint of the more proximal inversion; i.e., *bw* recombinants carrying *SD*, but neither the inversions nor the lethal and the complementary product, *cn* recombinants carrying SD^+, the lethal and both inversions. Finally, there has been recovered one case, *R(SD-5)-26*, of a double crossover; one crossover between *SD* and *cn* and the other between the distal breakpoint of the distal inversion and *bw* (i.e., a *cn* recombinant carrying *SD* but lacking the lethal and the inversions). This separation of *SD* from the lethal, it may be noted in passing, is a proof that *SD-5* does not carry a lethal allele of *SD*.

The number and the genetic constitution of the recovered recombinants is given in Table 1. Given there also, is the stability of each *SD*-bearing line.

The phenotype of unstable lines: All of the *SD* lines (originals and recombinants) have been repeatedly backcrossed to the "standard" *cn bw* stock, thus insuring uniformity in genetic background. Each *k* value determination is made from a standard backcross, *SD/ cn bw* single male by *cn bw* females. Determinations of this type were made for males carrying *SD-72*, and *SD*-bearing recombinants from *SD-72*, whose fathers showed a *k* value of 0.90 or greater. Histograms of the frequency distributions of *k* values from such males are shown in Figure 1. Similar distributions from *SD-5* type lines and recombinants from *SD-5* lines are given in Figure 2.

From these distributions, the phenotypic distinction among stable, semistable, and unstable lines is clear. It is evident, however, that the resolving power of these tests is rather low and they do not, by any means, exclude the possibility

TABLE 1

The number and constitution of the various types of recombinants between cn *and* bw *recovered from females heterozygous for an original SD-bearing second chromosome and the standard* cn bw *chromosome*

Representative symbol	Number recovered	Source	Constitution	*SD* allele	Stability state
R(SD)–10	4	*SD–72*	*cn In l⁺ bw⁺*	*SD⁺*	.....
R(SD)–4	4	*SD–72*	*cn⁺ In⁺ l⁺ bw*	*SD*	Semistable
R(SD)–6	3	*SD–72*	*cn⁺ In⁺ l⁺ bw*	*SD*	Unstable
R(SD–36)–1	1	*SD–36*	*cn⁺ In l bw*	*SD*	Unstable
R(SD–5)–15	8	*SD–5*	*cn In⁺ l⁺ bw⁺*	*SD⁺*	.....
R(SD–5)–18	2	*SD–5*	*cn⁺ In l bw*	*SD*	Semistable
R(SD–5)–17	6	*SD–5*	*cn⁺ In l bw*	*SD*	Unstable
R(SD–5)–34	2	*SD–5*	*cn In l bw⁺*	*SD⁺*	.....
R(SD–5)–32	1	*SD–5*	*cn⁺ In⁺ l⁺ bw*	*SD*	Unstable
R(SD–5)–26	1	*SD–5*	*cn In⁺ l⁺ bw⁺*	*SD*	Unstable

of levels of stability intermediate between any two of these. Indeed, it could be that every recombinant has a unique level of stability. Operationally, however, we are forced to restrict the classes to only these three.

Neither a semistable nor an unstable *SD* line can be made stable by selection. One semistable line, *R(SD)-4*, and one unstable line, *R(SD-36)-1*, have been selected for high k values (during routine stock transfers) for 30 generations or more without a change in the stability state. However, in any generation, if the sons of fathers that showed low k values are tested and compared with the sons of fathers that showed high k values, the distribution from the sons of high k fathers is usually—but not always—somewhat skewed towards high k as compared with the sons of low k fathers. In the next generation, however, k values of sons of high k fathers (or of low k fathers) are the same irrespective of the k value of the grandfather. Thus, it appears that selection is effective for one generation, but not thereafter.

Figure 3 shows the results of an experiment illustrating this point. A single heterozygous *R(SD-36)-1* male was chosen, and two of his sons selected. One of these exhibited a k value of 0.73 (his descendants then constitute a "low line"), the other a k value of 0.96 (thus beginning a "high line"). From this low line male, a single son, showing a k value of 0.53, was selected and crossed to a number of *cn bw* females to produce a large family of sons. The frequency distribution of k values for these sons is given in histogram 1 in Figure 3. The high line male was crossed to *cn bw* females, and a single son, showing a k value of 0.97, was selected and crossed to *cn bw* females to produce a large number of sons. The distribution of k values of these sons is shown in histogram 2 in Figure 3. It can be seen that in this case the two distributions are not different. Indeed, the unweighted mean k value is slightly higher for the low line than for the high line, although, as will be seen, this is not usually the case. From among these progenies, the sons of fathers showing low, intermediate, and high k values were selected, and their k values determined. The results are also shown in Figure 3 in

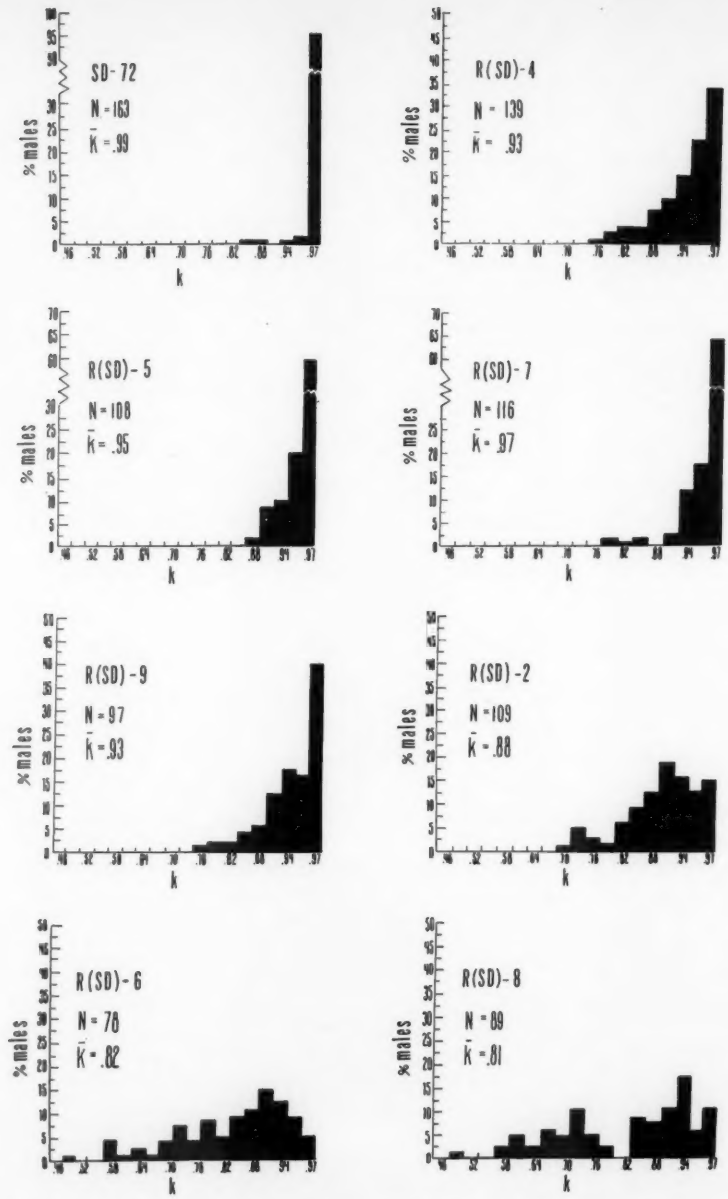

FIGURE 1.—The frequency distribution of *k* values from males heterozygous for *SD-72*, or an *SD*-bearing recombinant from *SD-72*, and *cn bw* by *cn bw* females.

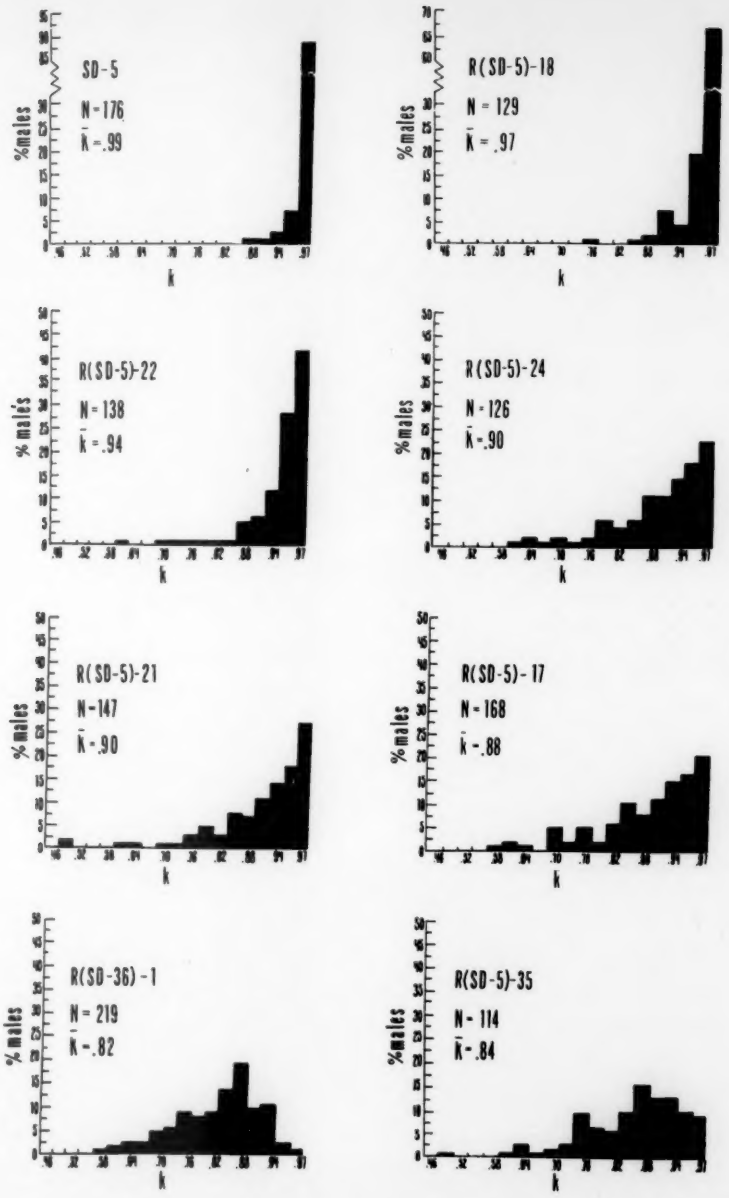

FIGURE 2.—The frequency distribution of k values from males heterozygous for *SD-5*, or an *SD*-bearing recombinant from *SD-5*, and *cn bw* by *cn bw* females.

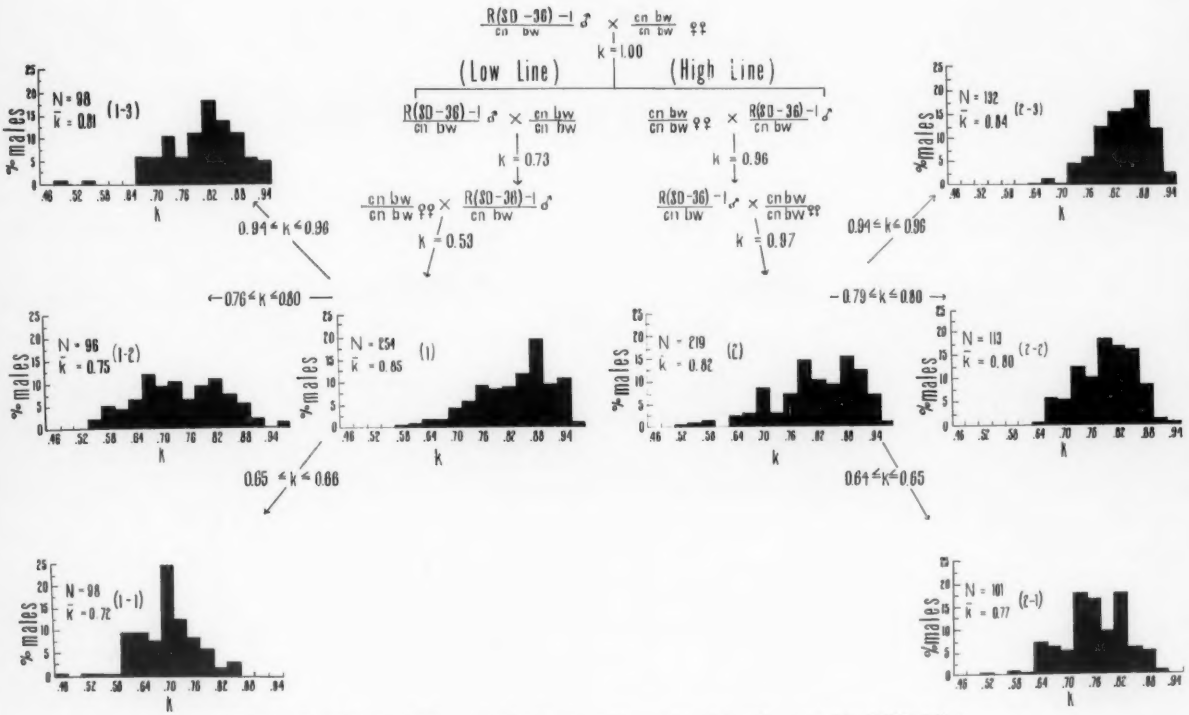

FIGURE 3.—The results from a selection experiment involving the unstable *R(SD-36)-1*.

histograms 1–1, 1–2, and 1–3 (for the low line) and 2–1, 2–2, and 2–3 (for the high line).

Here it can be seen that the mean k values do vary according to the parental k, but the designations "high line" and "low line" have no meaning. It is true that in this experiment, the unweighted mean k values from the high line are all slightly higher than the comparable sets from the low line, but in the previous generation the low line exhibited a higher mean k value than the high line. In any case, the distributions from any parental k value interval are not very different between the two lines.

In summary then, the k value of any male depends, to some extent, on the k value of his father, but, for any particular father, appears to be independent of the k value of his grandfather.

Tests for the possibility of influencing stability: In general, the behavior of *SD* is modifiable in many ways (SANDLER and HIRAIZUMI 1959). Stability, on the contrary, seems to be a relatively invariant property of an *SD* line. It has been shown that if any *SD*-bearing recombinant from *SD-72* is passed through a female (heterozygous, for example, with *In(2LR)Cy* to prevent crossing over), some such females produce sons, one half of which do not distort. Females that produce such atypical sibships are referred to as "conditioned", the phenomenon as conditional distortion (SANDLER and HIRAIZUMI 1959). It is also known that this phenomenon is suppressed in an attached-X and Y-bearing female (that is, such females produce only distorting sons). Although this phenomenon is observed in both semistable and unstable *SD* lines (in only those lines, however, derived by recombination from *SD-72*), no effect of either the sex of the *SD*-contributing parent or of an extra Y chromosome in the parent is observed on the phenomenon of instability itself. Evidence on these points is presented in Table 2 in which are shown the results from unstable *R(SD-36)-1*, heterozygous with *cn bw*, in males which received their *SD*-bearing chromosome from (1) a free-X female parent, (2) an attached-X and Y-bearing (*y f:* =/Y) female parent, or (3) a male parent. The same unstable behavior of *R(SD-36)-1* is evident in all three cases. The slight excess of low k values in the set in which *SD* was derived from the free-X female parent is almost certainly due to the slight response of the *SD-5* type lines and their derivative recombinants to the phenomenon of conditional distortion (SANDLER and HIRAIZUMI 1959).

It has been shown (SANDLER, HIRAIZUMI, and SANDLER 1959) that when *SD*-bearing chromosomes are made homozygous in a male, there is no evidence for either distortion or sterility. This result was interpreted to mean that an *SD*-bearing chromosome is completely insensitive to the action of another *SD* element. A possible ambiguity of this type of result is that each *SD* allele, in an *SD* homozygote, might distort the other with the same frequency, thus resulting in no net distortion. Unstable *SD* lines can be used in this connection, since in the case of a heterozygote for an unstable and a stable *SD* line, even if both *SD* chromosomes distort, there should result a distorted segregation ratio favoring the stable *SD* line. The test was made in the following way: two females of the constitution *R(SD-36)-1/cn bw* were each individually doubly mated to (a) *cn bw* males and (b)

TABLE 2

The frequency distribution of k *values from* R(SD–36)–1/cn bw *males in which the* SD*-bearing chromosome was derived from the male parent in one case, a free-X-bearing female parent in another case, and an attached-X and Y-bearing female parent in the final case. The percentage of tested males falling into each* k *value interval is given in the various columns*

	Source of *R(SD-36)-1*		
k	Male parent	Free-X female parents	$\overline{XX}$Y female parent
0.43	...	0.86	2.00
0.46	1.54	3.42	2.00
0.49	6.15	2.56	0.00
0.52	3.08	2.56	2.00
0.55	3.08	11.11	0.00
0.58	3.08	5.13	6.00
0.61	4.62	3.42	4.00
0.64	1.54	2.56	4.00
0.67	6.15	6.84	2.00
0.70	4.62	2.56	8.00
0.73	7.69	3.42	6.00
0.76	6.15	2.56	6.00
0.79	4.62	4.27	4.00
0.82	6.15	0.00	10.00
0.85	3.08	1.71	6.00
0.88	10.77	1.71	6.00
0.91	6.15	5.98	8.00
0.94	4.62	8.55	12.00
0.97	16.92	30.76	12.00
Total males	65	117	50
$\overline{k}$	0.81	0.79	0.80

SD-72/cn bw males (by allowing the sperm from the first mating to become exhausted and then mating to the next male). These same *SD-72/cn bw* males were also crossed individually to *cn bw* females. From the cross of *R(SD-36)-1/cn bw* female by *cn bw* males, *R(SD-36)-1/cn bw* males were recovered and tested to get a control frequency distribution of *k* values from this line. From the cross of *SD-72/cn bw* males by *cn bw* females, *SD-72/cn bw* males were recovered and tested to get a control distribution of *k* values from *SD-72*. Finally, the experimental males, *R(SD-36)-1/SD-72*, were recovered from the cross of *R(SD-36)-1/cn bw* females × *SD-72/cn bw* males, and tested. The results from these matings are given in Table 3. It can be seen that even though the distributions of *k* values for the two *SD* lines are very different, there is no evidence for any distortion in *SD-72/R(SD-36)-1* males. Thus, the conclusion that an *SD*-bearing chromosome is itself insensitive is confirmed here.

The *SD-72/R(SD-36)-1* heterozygotes derived in this way permit the exploration of another question. In certain SD^+ homozygotes, in which the SD^+ alleles differ from one another in their sensitivity to the distorting action of some stand-

ard *SD* allele, the *SD*+ alleles derived from these "homozygotes" are changed in sensitivity, each allele to a state more similar to that of the other allele. Such directed changes of state are referred to as *translocal modifications* (SANDLER and HIRAIZUMI 1959). It is of considerable interest, therefore, to determine whether instability is subject to such modification. Accordingly, the k values from *SD-72/cn bw* and *R(SD-36)-1/cn bw* males, that were the progeny of the *SD-72/R(SD-36)-1* males whose construction was given above, were determined. These results are given in Table 4. It can be seen that the distributions of k values of the stable *SD-72* and the unstable *R(SD-36)-1* have not been changed by virtue of the test chromosomes having been mutually heterozygous.

In summary, then, instability is a state characterizing an *SD* line which is not modifiable by those factors that modify the phenomenon of conditional distortion, nor are stability states subject to translocal modifications.

The genetic basis of instability: Thus far, every recovered *SD*-bearing recombinant has shown reduced stability. Furthermore, no unstable or semistable lines have been obtained from nonrecombinant *SD* chromosomes. These two facts would seem to imply that there is a stabilizing modifier somewhere on the right arm (since crossing over in IIL is normal) of chromosome II in the original, stable, lines which is lost by recombination and which loss results in a reduced

TABLE 3

The results from crosses of R(SD–36)–1/cn bw, R(SD–36)–1/SD–72, *and* SD–72/cn bw *males by* cn bw *females. The* R(SD–36)–1 *and* SD–72 *chromosomes were derived from the same males for all three tests*

	Type of male		
k	R(SD-36)-1/cn bw	R(SD-36)-1/SD-72	SD-72/cn bw
0.43	...	18.52	...
0.46	...	11.11	...
0.49	...	18.52	...
0.52	...	25.93	...
0.55	4.55	25.93	...
0.58	6.82	...	..
0.61	15.91	...	...
0.64	6.82	...	...
0.67	11.36	...	...
0.70	9.09	...	...
0.73	18.18	...	...
0.76	6.82	...	...
0.79	11.36	...	...
0.82	0.00	...	...
0.85	2.27	...	...
0.88	2.27	...	.
0.91	2.27	...	1.72
0.94	2.27	...	1.72
0.97	...	...	96.55
Total males	44	27	58
$\bar{k}$	0.71	0.51	0.99

TABLE 4

The results from crosses of R(SD–36)–1/cn bw *and* SD–72/cn bw *males by* cn bw *females. The* SD*-bearing chromosomes were derived from the* R(SD–36)–1/SD–72 *males recorded in Table 3*

	Type of male	
k	R(SD-36)-1/cn bw	SD-72/cn bw
0.52	1.61	...
0.55	1.61	...
0.58	1.61	...
0.61	3.23	...
0.64	6.45	...
0.67	8.06	...
0.70	9.68	...
0.73	12.90	...
0.76	9.68	...
0.79	17.74	...
0.82	8.06	...
0.85	6.45	1.64
0.88	6.45	0.00
0.91	6.45	0.00
0.94	...	0.00
0.97	...	98.36
Total males	62	61
$\bar{k}$	0.76	0.99

stability. Initially, such an hypothesis presents certain difficulties. The most pressing of these is that, if a modifying system is to explain both semistability and instability, then there must be at least two such loci conditioning the three possible states. For example, it might be that possessing both stabilizers conditions stability, possessing one stabilizer conditions semistability, and *SD* in the presence of neither stabilizer is unstable. Secondly, however, it appears that these stabilizers cannot occupy the same positions in *SD-5* and *SD-72* for the reason that both stabilizers must be distal to the distal inversion in *SD-5* (in order to recover both semistable and unstable recombinants in those cases in which the crossover occurred between the distal breakpoint of the distal inversion and the locus of *bw*), whereas at least one of the stabilizers must be proximal to the inversion in *SD-72* (in order to recover both unstable and semistable states among those recombinants in which the crossover took place proximal to the *SD-72* inversion).

In order to circumvent these difficulties, experiments were performed to see whether it is possible that semistable lines do not differ from unstable lines by a modifier. Three different selection experiments were carried out for the purpose of selecting an unstable line from a semistable one, passing the semistable line through males only so that the possibility of further crossing over is eliminated. The semistable line chosen for these experiments was *R(SD)-4*.

The first such experiment involved choosing a single *R(SD)-4/cn bw* son, of a *R(SD)-4/In(2LR)Cy* father, which son showed a k value of 0.85. The Curly in-

version contains long inversions in both the left and right arms of chromosome II. The frequency distribution of k values of the sons of this *R(SD)-4/cn bw* male were determined, and from these X_1, progeny were selected from parental males giving high and low k values and these progeny tested for their k value distributions. These results are shown in Table 5. It is clear that the descendants of the selected male are much more unstable than the standard, semistable, *R(SD)-4*.

The second experiment involved the selection of two *R(SD)-4/cn bw* males from a *R(SD)-4/cn bw* father. The distribution of k values among the progeny of these two males and from among the X_2, with selection again for high and low k values among the X_1, was determined. These results are presented in Table 6. Again it is evident that the line so selected is less stable than the original *R(SD)-4*.

In the final selection experiment of this type, the same procedure was followed except that in this case the original *R(SD)-4/cn bw* males were obtained from a homozygous *R(SD)-4* father. The results of these experiments are given in Table 7. Once again the instability of the line selected is clear.

It should be noted, finally, that unstable lines can also be derived from the semistable *R(SD)-4* line passed through females. The evidence on this point is presented in Table 8. Here, a single *R(SD)-4/cn bw* male, giving a k value of 0.68, was selected from a *R(SD)-4/cn bw;* attached-X and Y-bearing mother. Two gen-

TABLE 5

The results of three generations of tests indicating instability among the descendants of a single R(SD)–4/In(2LR)Cy *male. Every testcross is of a* R(SD)–4/cn bw *male by* cn bw *females*

		Generation				
		X_1	X_2		X_3	
k	Parental k	0.85	$0.91 \leq k \leq 0.96$	$0.66 \leq k \leq 0.73$	$0.83 \leq k \leq 0.94$	$0.61 \leq k \leq 0.69$
0.49		...	...	...	0.95	
0.52		...	...	...	0.00	1.75
0.55		...	...	...	0.00	0.00
0.58		...	...	...	0.00	1.75
0.61		...	1.69	...	0.00	1.75
0.64		5.56	0.00	3.23	0.00	7.02
0.67		0.00	3.39	3.23	0.00	1.75
0.70		0.00	0.00	0.00	1.90	5.26
0.73		11.11	3.39	12.90	0.00	3.15
0.76		5.56	0.00	6.45	7.62	8.77
0.79		11.11	5.08	3.23	0.95	12.28
0.82		16.67	10.17	12.90	5.71	7.02
0.85		11.11	8.47	9.68	3.81	14.04
0.88		11.11	8.47	16.13	8.57	7.02
0.91		5.56	18.64	6.45	14.29	17.54
0.94		16.67	13.56	3.23	20.00	10.53
0.97		5.56	27.12	22.58	36.19	...
Total males		18	59	31	105	57
$\bar{k}$		0.85	0.90	0.86	0.92	0.82

TABLE 6

The results of two generations of tests indicating instability among the descendants of two R(SD)-4/ cn bw *males. Each test is a cross of a single* R(SD)-4/ cn bw *male by* cn bw *females*

		Generation		
		X_1	X_2	
k	Parental k	$0.78 \leq k \leq 0.95$	$0.89 \leq k \leq 1.00$	$0.57 \leq k \leq 0.73$
0.55		2.50	...	...
0.58		2.50	...	6.82
0.61		2.50	...	2.27
0.64		5.00	...	2.27
0.67		5.00	...	2.27
0.70		0.00	...	0.00
0.73		7.50	1.52	4.55
0.76		5.00	4.55	6.82
0.79		5.00	1.52	0.00
0.82		15.00	4.55	2.27
0.85		5.00	7.58	9.01
0.88		27.50	13.64	15.91
0.91		2.50	10.61	15.91
0.94		12.50	15.15	9.01
0.97		2.50	40.90	22.72
Total males		40	66	44
$\bar{k}$		0.83	0.93	0.84

erations of descendants from this male were tested for distortion, with selections made in the same way as above. It is clear that an unstable line has been derived from a semistable one as in the case of the passage of *R(SD)-4* through the various kinds of males.

Thus, for all cases, it appears that unstable *SD* lines can be selected from a semistable line without the necessity of crossing over. It may be concluded, therefore, that the semistable *SD* state does not differ from the unstable *SD* state by a modifier.

Since this is so, the question of stabilizing modifiers resolves itself simply to a consideration of a single modifier that conditions the difference between stability and lack of stability—the latter being either semistability or instability. This stabilizing modifier, *St(SD)*, must, moreover, be located at the tip of the right arm of chromosome II, very close to (and, indeed, probably distal to) the locus of *bw*, for the reason that no *SD*-bearing recombinant is stable. It must be, therefore, that every SD^+-bearing recombinant carries *St(SD)*. Two tests for the presence of this stabilizer were performed. In one, the semistable *R(SD)-4* was made heterozygous with an SD^+-bearing recombinant which must, under this hypothesis, carry *St(SD)*, and the stability of this *R(SD)-4* heterozygote measured. Secondly, the tip of IIR carrying the normal allele of *bw*, and therefore *St(SD)*, from each of four SD^+-bearing recombinants (from *SD-5*) was transferred to *R(SD)-4*, by crossing over, and the stability of such *R(SD)-4*, bw^+, *St(SD)* chromosomes determined. The results of these tests are presented in Table 9, along with the stand-

ard distributions of *R(SD)-4* and *SD-5*. It can be seen that, in the presence of the tip of the right arm of chromosome II, from the original, stable, *SD*-bearing chromosome, in either coupling or repulsion with *SD*, *SD* is completely stable.

Thus, it is possible to conclude that (1) in the presence of *St(SD)*, located at the tip of IIR, *SD* is completely stable; (2) in the absence of this modifier, *SD* is either semistable or unstable—the latter state being derivable from the former (but not *vice versa*) by selection without further crossing over.

The effect of St(SD) *on unstable* SD *lines:* The existence of *St(SD)* was inferred from the modifier's ability to stabilize the semistable *SD* state. The following experiments were performed for the purpose of examining the effect of *St(SD)* on the unstable *SD* state. The unstable line chosen for these experiments was *R(SD-36)-1*. A number of *R(SD-36)-1/cn bw* sibling males were crossed both to *R(SD-5)-15/In(2LR)Cy* females and to *cn bw* females [*R(SD-5)-15* carries *St(SD)* and is marked by *cn*]. From among the progeny, *R(SD-36)-1/cn bw* males and *R(SD-36)-1/R(SD-5)-15* males were collected and crossed to *cn bw* females so that the distribution of k values could be determined. These distributions are presented in histograms C–1 and E–1, respectively, in Figure 4.

TABLE 7

The results of two generations of tests indicating instability among the progeny of a single homozygous R(SD)-4/R(SD)-4 *male. Each test is a single* R(SD)-4/cn bw *male by* cn bw *females*

		Generation		
		X_1	X_2	
k	Parental k	$0.58 \leq k \leq 0.89$	$0.89 \leq k \leq 0.98$	$0.71 \leq k \leq 0.78$
0.43		2.44	...	...
0.46		0.00	...	...
0.49		0.00	...	...
0.52		0.00	...	2.04
0.55		0.00	...	2.04
0.58		2.44	1.56	4.08
0.61		0.00	3.13	4.08
0.64		0.00	0.00	2.04
0.67		2.44	0.00	8.16
0.70		9.76	3.13	8.16
0.73		2.44	4.69	2.04
0.76		7.32	6.25	6.12
0.79		2.44	6.25	8.16
0.82		9.76	10.94	8.16
0.85		7.32	7.81	6.12
0.88		7.32	18.75	18.37
0.91		7.32	17.19	12.24
0.94		12.19	7.81	6.12
0.97		26.83	12.50	2.04
Total males		41	64	49
$\bar{k}$		0.86	0.87	0.80

TABLE 8

The results of two generations of tests indicating instability among the descendants of a single R(SD)-4/ cn bw *male derived from a* R(SD)-4/ cn bw; *attached-X/Y female. Each test is a cross of a single* R(SD)-4/ cn bw *male by* cn bw *females*

		Generation		
		X_1	X_2	
k	Parental k	$k=0.68$	$k=0.97$	$0.57 \leq k \leq 0.77$
0.55		10.00	...	4.00
0.58		10.00	5.88	2.00
0.61		0.00	5.88	8.00
0.64		20.00	5.88	6.00
0.67		0.00	5.88	2.00
0.70		0.00	0.00	2.00
0.73		0.00	5.88	10.00
0.76		10.00	17.64	4.00
0.79		10.00	0.00	8.00
0.82		0.00	0.00	8.00
0.85		0.00	0.00	8.00
0.88		0.00	0.00	12.00
0.91		10.00	29.41	14.00
0.94		0.00	23.53	10.00
0.97		30.00	...	2.00
Total males		10	17	50
$\bar{k}$		0.79	0.82	0.81

TABLE 9

The stability of a semistable line, R(SD)-4, *onto which the original tip of IIR carrying the normal allele of* bw *has been replaced, and also in which the original tip is carried in repulsion. Given here also are the standard distributions of* R(SD)-4 *and* SD-5

	Type of male			
k	SD-5/cn bw	R(SD)-4/cn bw	R(SD)-4, St(SD)/cn bw	R(SD)-4/ R(SD)-15, St(SD)
0.73	...	0.72	...	...
0.76	...	2.88	...	...
0.79	...	4.32	1.30	...
0.82	...	4.32	0.00	...
0.85	2.14	6.47	0.52	...
0.88	2.14	10.07	1.55	...
0.91	2.84	15.11	3.11	1.33
0.94	5.68	21.58	9.33	6.67
0.97	88.07	33.81	84.20	92.00
Total males	176	139	386	75
$\bar{k}$	0.99	0.93	0.98	0.99

It can be seen that whereas *St(SD)* does not stabilize *R(SD-36)-1*, the control distribution does differ from the experimental set in that at least the high k values in the latter are extremely high. Thus, almost 40 percent of *R(SD-36)-1/St(SD)* males show k values of 0.97 or more, while no k values in this interval are observed in the controls. Although unstable distributions vary considerably, this difference is undoubtedly significant because the very same parental males contribute, in proportion, to the experimental and control distributions. In this generation *R(SD-36)-1/R(SD-5)-15* males exhibiting $0.98 \leqq k \leqq 1.00$ and also $0.62 \leqq k \leqq 0.70$ were chosen and crossed both to *cn bw* females and to *R(SD-5)-15/In(2LR)Cy* females. From the cross to *cn bw* females, *R(SD-36)-1/cn bw* males were collected and tested (giving "high" and "low" control distributions); from the cross to *R(SD-5)-15/In(2LR)Cy* females, *R(SD-36)-1/R(SD-5)-15* males were collected and tested (giving the two types of experimental distributions). The results are presented in the last four histograms in Figure 4.

In the control sets, C–L and C–H, the usual parent-offspring correlation in unweighted mean k values is apparent. Furthermore, these control distributions seem fairly typical (although perhaps somewhat high). The experimental sets, on the contrary, are most certainly high as compared with the usual behavior of *R(SD-36)-1* (see Figure 3) indicating that *St(SD)* does change the characteristic unstable distribution. However, in no case is the distribution stable. Thus, whereas *St(SD)* can completely stabilize semistable *SD* lines, it enhances—but does not succeed in stabilizing—the distorting action of unstable *SD lines*.

The case of R(SD-5)-26: The one recovered recombinant that carries both the mutant allele of *cn* and *SD*, *R(SD-5)-26*, also carries the normal allele of *bw*. This recombinant apparently arose from a double crossover, one exchange between *SD* and *cn*, the other between *bw* and the distal breakpoint of the distal inversion (see Table 1). Since the normal allele of *bw* and *St(SD)* have been inseparable in all of the experiments reported thus far, this recombinant ought to carry the modifier and thus be stable. The frequency distributions of k values, selecting parents exhibiting both high and low k values, is presented in Table 10. It is evident that *R(SD-5)-26* is extremely unstable—exhibiting, as a matter of fact, a distribution of k values lower than that of any other line.

There are two possible reasons for this. It may be that the distal crossover giving rise to *R(SD-5)-26* took place between *St(SD)* and bw^+ resulting in a recombinant that does not carry the modifier. It is, on the other hand, possible that *SD* itself has been changed by virtue of a crossover in its immediate vicinity. This latter seems not unreasonable because (1) it would be strange if, in this one exceptional recombinant, the modifier had separated from bw^+, (2) because it has been shown (SANDLER and HIRAIZAMI 1959) that crossing over in the vicinity of *SD* is very low which could mean that a high proportion of the occasional recombinants that do occur in the region of *SD* result in a change at the *SD* locus itself, and (3) because the distribution of k values is lower for this recombinant than those characteristic of the other unstable lines.

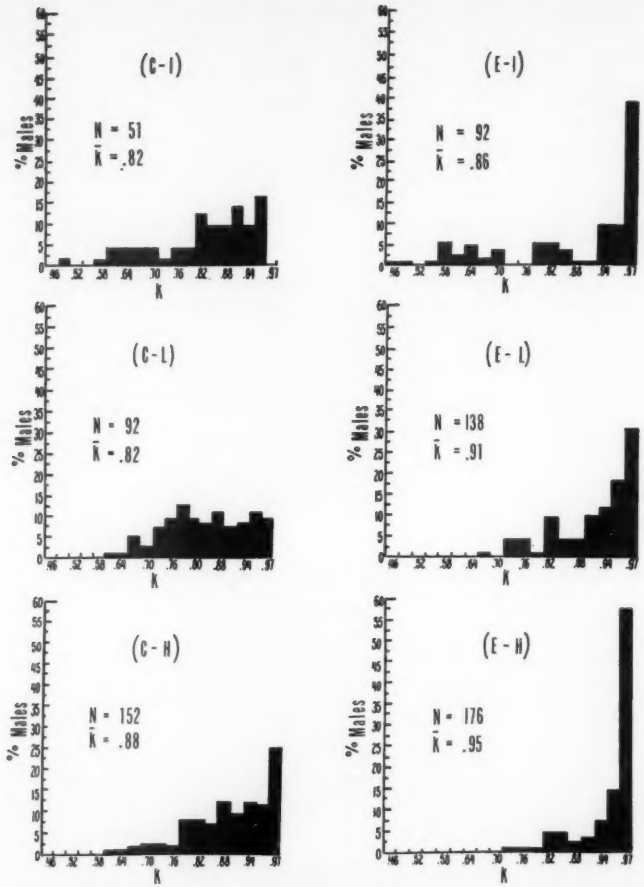

FIGURE 4.—The effect of *St(SD)* on the behavior of the unstable *R(SD-36)-1*. "C" stands for control, "E" for experimental, "L" for low line, and "H" for high line. C–1 and E–1 are control and experimental sets prior to selection (see text).

DISCUSSION

It is clear that stability depends on the presence of a modifier, *St(SD)*. In the absence of *St(SD)*, *SD* is either semistable or unstable, the latter state being derivable from the former (but not *vice versa*) by selection. It is, of course, possible that all recombinants losing *St(SD)* first become semistable and then, spontaneously, pass into the unstable state. Our unstable lines would then be cases in which this happened during the time the lines were being established.

Instability (and presumably semistability) is not simply phenotypic male to

TABLE 10

The frequency distribution of k *values from crosses of* R(SD-5)-26/ cn bw *males by* cn bw *females. Set A is tested males from fathers showing* k *values between 0.96 and 1.00; set B is from fathers whose* $0.49 \leq k \leq 0.79$

k	Set A	Set B
0.43	...	2.03
0.46	...	3.66
0.49	2.88	5.28
0.52	2.16	6.09
0.55	1.44	8.94
0.58	5.04	7.72
0.61	6.47	6.50
0.64	9.35	6.50
0.67	5.76	5.70
0.70	7.91	6.91
0.73	6.47	2.85
0.76	5.04	3.66
0.79	5.04	4.47
0.82	10.07	4.88
0.85	7.19	3.66
0.88	8.63	2.44
0.91	10.79	4.47
0.94	3.60	8.50
0.97	2.16	5.70
Total males	139	246
$\bar{k}$	0.76	0.71

male variation as evidenced by the heritability of high and low k values for one generation. Thus, it must be that instability involves two or more distinct *SD* states. Furthermore, an *SD* state may characterize either a male or an individual cell. That is, it may, on the one hand, be that every male, in an unstable *SD* line, has a characteristic k value. In this case there must be a high mutability from state to state in every generation since the frequency distribution of k values does not become more stable with selection. On the other hand, it could be that an *SD* state characterizes, not a male, but an individual cell. Thus, we may suppose that unstable *SD* lines are composed of *SD* alleles in, for example, two states; one characterized by high k value (i.e., very likely to distort), the other characterized by a low k value. In every gonial cell generation, there is some probability of a change of state such that the total probability of an *SD* allele entering meiosis in the alternative state to that which the male in question received from his father is close to, but less than, one half (since the number of gonial generations is fairly large, these can be any of a large number of mutation rates per cell generation). Thus, each tested male represents a population of *SD*-bearing chromosomes, and the particular k value he exhibits depends on the relative proportions of the two distorting states going through meiosis. Because the total probability of a "gonial mutation"—in this sense—is less than one half, there will be some correlation between father and son; but because there are only two states, there is no effect of

the grandparental k value. Instability according to this model then, means the change from one state to the other at a high rate (in total).

Semistability, on this model, can be either (1) a low rate of change to the low k state or (2) a low k state characterized by a higher k value than the low k state in the unstable lines. It is, of course, possible to postulate any number of stability states of chromosomes consistent with the data. As the number of such postulated states becomes large, the system becomes equivalent to supposing that every particular cell has its own probability distribution of k values, which distribution depends upon the parental k value only.

In summary, then, if an *SD* state characterizes a male, then there must (a) be many states, and (b) be a high state to state mutation rate per generation. If, on the other hand, an *SD* state characterizes a cell, then (a) there may be as few as two states, and (b) the mutation rate per cell generation can be fairly low. It is not possible, at this time, to distinguish between these two alternatives.

Evidently, semistable lines differ from unstable ones. This is evident both because the distribution of k values is different between the two states and also because *St(SD)* stabilizes the semistable state but not the unstable one. Furthermore, the high k states in unstable lines are most probably, themselves, different from the high k states in semistable or stable lines, since even the highest k states cannot be stabilized by *St(SD)*. The action of *St(SD)* can be either that of increasing the k value associated with any state of *SD* or it can be to decrease the mutation rate to the lower k states.

The position of *St(SD)* is of some interest. The *SD* locus, itself, is located in or near the proximal heterochromatin of chromosome II. Moreover, various SD^+ lines are known that differ in their sensitivity to the distorting action of *SD*. The basis of these differences is located at the SD^+ locus and, therefore, presumably also in the heterochromatin. Thus, it seems conceivable that those factors that affect segregation-distortion are all heterochromatic. The stabilizing modifier, however, is located at the tip of IIR. It is therefore, interesting to note that various lines of evidence suggest that the tips of chromosomes in *D. melanogaster* are heterochromatic (for arguments see MULLER 1938). It may be, therefore, that if the other modifiers of *SD* that are known are located in heterochromatin, then the generality that *SD* is affected by heterochromatin only will become justified.

Finally, the evidence presented in this report suggests that *SD* has existed in nature for a fairly long time. This is so first because the modifying system of instability must have had time to evolve. Secondly, it has been noted that *SD* in nature is always associated with efficient crossover suppressing inversions (HIRAIZUMI, SANDLER and CROW 1960) in IIR. Certainly, one function of these inversions must be to keep *St(SD)* linked to *SD* and thus insure uniform high k values. This system of inversions is, however, different among the different *SD* lines. These differences also suggest a long evolutionary history of *SD*.

SUMMARY

1. The *SD* lines collected in nature show unweighted mean k values (proportion of *SD* chromosomes transmitted by heterozygous males) of about 0.99 with very little male to male variation; such lines are referred to as "stable".

2. This stability is due to a modifier, *St(SD)*, located at the tip of the right arm of chromosome II.

3. In the absence of *St(SD)*, male to male variation is increased. This variation may be fairly wide (in which case the line is termed "semistable") or very wide (in which case the line is referred to as "unstable").

4. No line can be made more stable by selection, but a semistable line can become unstable.

5. Various possible mechanisms to explain instability and also the action of *St(SD)* are considered.

6. The existence of a stabilizing modifier and a system of inversions for keeping *St(SD)* and *SD* linked, in the *SD* system, implies that *SD* has existed in nature for a fairly long time.

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LINKAGE OF GENETIC MARKERS IN PHAGES T2 AND T4

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IN organisms that undergo meiosis, genetic linkage may be defined as the appearance (among the gametes produced by an F_1 of a dihybrid cross) of a frequency of genotypes different from that predicted on the basis of a free assortment of the alleles at meiosis. This criterion of linkage is not directly applicable in the case of bacteriophage because a cross cannot be performed in an analogous fashion. Indeed, the production of recombinant phage by a mixedly infected bacterial cell can be understood only if it is approached as a problem in population genetics (VISCONTI and DELBRÜCK 1953; HERSHEY 1958).

The measurements made in a phage "cross" are (1) the measurements of genotype frequencies prior to any opportunities for recombination, and (2) measurements of genotype frequencies in the population at later times, after the appearance of infectious phage within the cell. The initial constitution of the population of mating entities (vegetative phage) can be controlled by adjusting the frequencies of the genotypes which initiate the infection. The constitution at any time later, however, may depend on many factors. The number of opportunities for genetic recombination ("matings"), the way in which these matings are distributed in space (the degree of panmixia) and time, the number of vegetative phage particles participating in each mating, and the probability of recombination per mating, as well as the initial genotype frequencies, all may influence the subsequent genetic constitution of the population (HERSHEY 1958; BRESCH 1959).

It is clearly as inappropriate to apply the criterion of nonrandom association of genetic markers among the progeny of a phage "cross" as indicative of linkage as it is to assume that random or near-random association implies nonlinkage. A definition of linkage for two factors which relates to the probabilities of the possible genotypes of vegetative phage emerging from a single mating act would be more to the point. Such a definition would be awkward, however, unless one made specific assumptions about the mechanism of recombination (break-reunion or copy-choice), about the number of vegetative phage particles participating in a mating event, and about the degree to which the participating phages are able to share in the production of any recombinant. Such assumptions would at present be based on weak or nonexistent experimental observations. Furthermore, such

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a definition would have limited operational usefulness, since particles emerging from a single act cannot be directly examined.

In terms of the assortment of alleles at three loci, one can make a definition of linkage that is completely free of assumptions concerning either the kinetics or the mechanism of recombination.

In a phage cross involving linked loci, of the type $ab \times a^+b^+$, the recombinant classes, ab^+ and a^+b are examined for the frequency with which they include one of the two alleles at a third locus whose linkage to *ab* is in question. If the third locus, *c*, is not linked to *ab*, it stands, by definition, in a symmetrical relationship to *a* and *b*,

$$\begin{matrix} c \\ a\ b \end{matrix}$$

and the choice of recombinant class between the *a* and *b* loci will have no influence on the occurrence of *c*.

If locus *c* is linked to *ab*, however, then its relationship is no longer symmetrical, that is, the complete genotype may be written either as

$$\frac{a\ \ b \qquad\qquad c}{}$$

or as

$$\frac{c \qquad\qquad a\ \ b}{}$$

and the cross to test linkage is either

(1)
$$\frac{a\ \ b \qquad\qquad c}{}$$

$$\frac{}{a^+\ b^+ \qquad\qquad c^+}$$

or

(2)
$$\frac{c \qquad\qquad a\ \ b}{}$$

$$\frac{}{c^+ \qquad\qquad a^+\ b^+}$$

In cross (1) a single mating act should more often lead to the formation of ab^+c^+ than to the formation of ab^+c because only one event is required for the former, while two are necessary for the latter. This is true independently of whether the events are breaks and reunions, or switches from one parent to the other during the course of replication, and independently of whether mating is pairwise or groupwise. For cross (2) the frequencies are reversed.

In both crosses, the frequencies of $(ab^+)c^+$ and $(ab^+)c$ tend towards the same equilibrium value as the number of rounds of mating increases. Two variables can be manipulated to aid in maintaining the difference in frequencies. The opportunities for successive matings can be minimized by premature lysis, and the crosses can be performed with an unequal multiplicity of the two parents.

In practice, two crosses are performed: Cross I with $abc > a^+b^+c^+$ and Cross II with $abc < a^+b^+c^+$ (Figure 1), and bacteria are prematurely lysed in order to

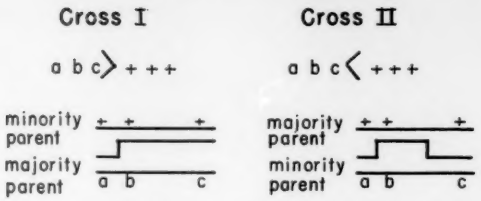

FIGURE 1.—The types of crosses used to detect linkage.

examine the phage population when the number of rounds of mating is small. The relative multiplicities of the majority and minority parents are the same for each cross. In each case ab^+ recombinants are selected and *among these* the frequency of the *c* allele contributed by the minority parent is measured. The frequency desired for Cross I is $\frac{ab^+c^+}{ab^+c + ab^+c^+}$; that for Cross II is $\frac{ab^+c}{ab^+c + ab^+c^+}$. If *c* is not linked to *ab*, the frequencies would depend solely on the relative multiplicities of the majority and minority parents, and should thus be identical for the two crosses. In the case of linkage, however, the frequency for Cross I (where the desired class may be the result of one event) should be higher than the frequency for Cross II (where two events are required). This criterion of linkage is entirely independent of the mechanism of mating or replication.

MATERIALS AND METHODS

Phage strains T2H *r*1, *r*2, and *r*7 were obtained from DR. A. D. HERSHEY; T4D *r*47, *r*48, and *tu*45 were obtained from DR. A. H. DOERMANN; and *r*73 was isolated in the course of this study.

Bacterial strains *Escherichia coli* H and S were obtained from A. D. HERSHEY, BB from G. STENT, K-12 (λ) from J. WEIGLE, and B from S. E. LURIA.

Experiments with T2

Media.—Broth: Bacto peptone, 10 gm; Bacto beef extract, 3 gm; sodium chloride, 5 gm; glucose, 1 gm; H_2O, 1 liter. Bottom agar: Bacto agar, 10 gm; Bacto tryptone, 10 gm; NaCl, 8 gm; sodium citrate, 2 gm; glucose, 1 gm; H_2O, 1 liter. Top agar: Has the same composition but contains only 0.7 percent agar. Buffer: Na_2HPO_4, 3 gm; KH_2PO_4, 1.5 gm; NaCl, 4 gm; K_2SO_4, 5 gm; gelatin, 0.01 gm; $MgSO_4$, 10^{-3} M; $CaCl_2$, 10^{-4} M; redistilled water, 1 liter. M-9: Na_2HPO_4, 7 gm; KH_2PO_4, 3 gm; NH_4Cl, 1 gm; H_2O, 1 liter.

Stocks were prepared on BB in M-9, and in the case of double *r* mutants were concentrated by centrifugation.

Crosses were performed on H grown in broth to a titer of about 1×10^8 per ml, washed and resuspended in buffer, and aerated for 45 to 60 minutes.

Premature lysis was accomplished by dilution into broth saturated with chloroform (SÉCHAUD and KELLENBERGER 1956).

Plating was on S or else on K-12 (λ). The bottom agar described below was used for the latter.

Experiments with T4

Media.—Broth: Bacto tryptone, 10 gm; NaCl, 5 gm; glucose, 10 gm; (added after autoclaving); H_2O, 1 liter. Bottom agar: Bacto agar, 10 gm; bacto tryptone, 10 gm; NaCl, 5 gm; H_2O, 1 liter. Top agar: Has the same composition but contains 0.7 percent agar.

Stocks were prepared on BB, by the plate method (SWANSTROM and ADAMS 1951), and were purified by centrifugation.

Crosses were performed on B grown in broth to a titer of about 1×10^8 per ml, washed and resuspended in buffer, and aerated for 45 to 60 minutes. Tryptophane (20 γ/ml) was added immediately before infection. Usually more than 90 percent of bacteria survived as infective centers.

Premature lysis is accomplished by dilution into broth saturated with chloroform.

Platings were performed on B or on K-12 (λ).

EXPERIMENTS

Linkage of r7 and r1 in T2: We chose two closely linked factors, *r*2 and *r*7, and tested for linkage to *r*1, a factor previously considered unlinked. Mutants *r*2 and *r*7 are members of the *r*II group (BENZER 1955) and do not form plaques on *E. coli* strain K-12 (λ) (=K). Only *r*2+ *r*7+ recombinants among the progeny of a cross of *r*2 × *r*7 phage will give rise to plaques when plated on K, and these plaques can readily be classified as *r*1 or *r*1+ by inspection.

Crosses of *r*2 *r*7+ *r*1 × *r*2+ *r*7 *r*1+ were performed with multiplicities of 1: ⩾10 or ⩾10:1 phage particles of each type per bacterium (Crosses Ia and IIa, Figure 2). In order to recognize any selective effects due to the presence of the *r*1 or the *r*1+ allele, similar crosses were performed with the positions of the two *r*1 alleles reversed (Crosses Ib and IIb, Figure 2).

The bacteria in which the crosses were performed were lysed as soon as they contained measurable numbers of intracellular phage particles. Differences in the frequencies of recombinant types are expected to be greatest at this time. Samples were also lysed at various later times, to observe the changes in recombinant frequencies to be expected because of repeated matings. The results are shown in Figure 2 where the frequency of the *r*1 allele of the minority parent, among *r*2+ *r*7+ recombinants, is plotted for each of the four crosses. The frequencies of the minority allele were different in Cross I and Cross II, indicating linkage among the three markers. The order of the markers is *r*2–*r*7–*r*1. Reversing the positions of the *r*1 alleles did not lead to important changes in frequencies, an indication that no strong selective effect can be ascribed to either of the *r*1 alleles. In experiments performed on any one day the difference in recombinant frequencies between the two types of crosses was more consistent than appears from the figure.

The efficiency of plating (e.o.p.) of T2 *r*II+ on K is 0.1–0.3 of that on B. The

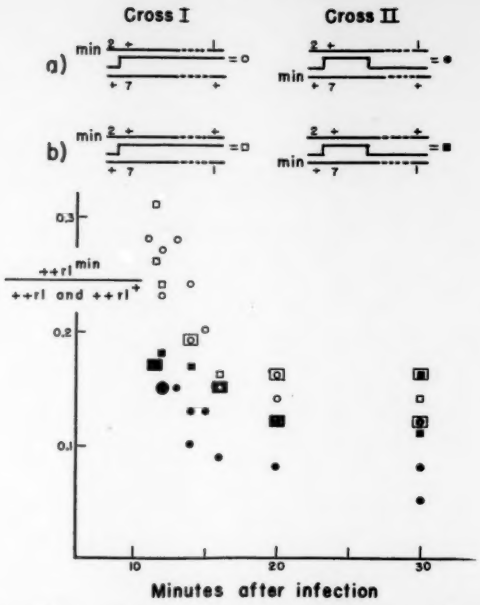

FIGURE 2.—Linkage between *r*1 and *r*7 in T2. Four crosses were performed as illustrated in the figure, where min indicates the minority parent. Results are plotted as frequencies of the *r*1 allele, contributed by the minority parent among recombinants ($r2^+$ $r7^+$) with respect to *r*2 and *r*7, for varying times of lysis (infection at time zero). The different symbols refer to the different crosses that are illustrated. Large symbols, or superimposed symbols, represent several points.

low e.o.p. does not influence the results described above since the e.o.p. of *r*II+ *r*1 and *r*II+ *r*1+ on K are identical (compare also the results of Crosses a and b, Figure 2). The low e.o.p. does, however, interfere with a precise determination of the frequency of *r*II+ phage among the progeny at various times of lysis. A determination of this frequency seemed necessary in order to compare the rates of replication and mating in crosses of types I and II. Crosses were therefore performed with T4, since the e.o.p. of T4 *r*+ on K is identical to that on B.

Linkage of r47 and r48 in T4: Experiments identical to the ones described above were performed with phage T4. The linkage of the *r*II mutants *r*47 and *r*73 to *r*48 (a mutant linked to the *r*1 of T2) was examined.

The types of crosses made and the results obtained are shown in Figure 3A. There was again a clear-cut distinction between crosses of types I and II, indicating linkage in the order *r*73–*r*47–*r*48. It seemed possible that the differences in frequencies of recombinant types measured in crosses of types I and II were due to different rates of replication and mating. To evaluate this possibility, the frequency of *r*73+ *r*47+ recombinants among the total progeny was measured in one cross of type I and one of type II (Figure 3B). The fact that the frequency of

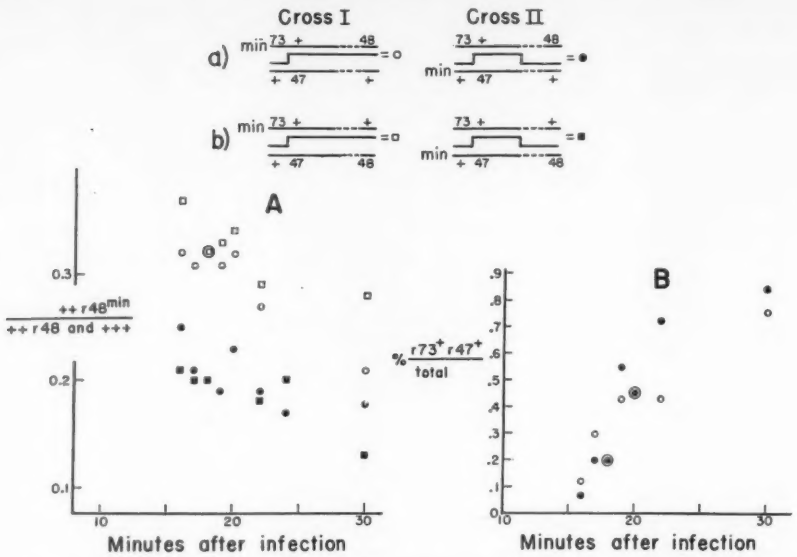

FIGURE 3.—Linkage between *r*47 and *r*48 in T4. Four crosses were performed, as illustrated in the figure, where min indicates the minority parent. The different symbols refer to the different crosses that are illustrated. Large symbols, or superimposed symbols, represent several points. Results in A are plotted as frequencies of the *r*48 allele, contributed by the minority parent among recombinants ($r73^+$ $r47^+$) with respect to *r*73 and *r*47, for varying times of lysis (infection at time zero). The percents of recombinants $r73^+$ $r47^+$ in the total population are plotted in B.

recombinants at various times of lysis was similar for the two crosses indicated that there were no large differences in rates of mating.

A comparison of the order rII *B cistron,* rII *A cistron and* r1 *(*r48*) in T2 and T4:* The mutants *r*47 and *r*73 of T4 belong to cistrons A and B, respectively (BENZER 1955; EDGAR 1958). It could be shown, by spot-test crosses against a set of standard T4 deletions as described by BENZER and FREESE (1958), that *r*7 of T2 belongs to the A cistron, and *r*2 of T2 to the B cistron. The order B cistron—A cistron—*r*1 (*r*48) is thus the same in T4 and T2.

Linkage of r47, r48, *and* tu45 *in T4:* We examined the linkage of *r*47 and *r*48, already shown to be linked, to *tu*45 previously considered unlinked to the others.

The design of the experiments was similar to those previously discussed. Crosses of *r*47 *r*48 *tu*45 × *r*47+ *r*48+ *tu*45+ were performed at a multiplicity of 1:10 and 10:1, and, to control possible selection, the cross was repeated with the position of the *tu* alleles reversed. The progeny were plated on K, and the *r* plaques appearing were classified as *tu* 45 or *tu*45+ (Figure 4). The *r* plaques on K represented *r*47+ *r*48 recombinants; classifying these as *tu* or *tu*+ distinguishes the single-recombinant from the double-recombinant types. It is clear from the results

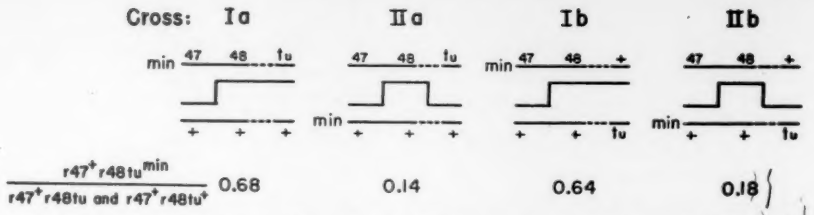

FIGURE 4.—Linkage between *r*48 and *tu*45 in T4. Four crosses were performed, as illustrated. The frequencies of the *tu*45 allele, contributed by the minority parent, among recombinants (*r*47$^+$ *r*48) with respect to *r*47 and *r*48, in cultures lysed 11½ minutes after infection, are presented.

presented in Figure 4 that there is strong linkage between *r*48 and *tu*45 and that the order is *r*47–*r*48–*tu*45.

DISCUSSION

Our data indicate that the known genetic markers of phages T2 and T4 are linked as had already been found for the smaller phages. Thus all the known bacteriophages have a continuous genetic map, and therefore, presumably, a continuous genetic structure. This continuity simplifies the problem of the assembly of all of the genetic material of a bacteriophage into one particle during the course of maturation.

Our results confirm the association of two groups previously considered unlinked that was independently described by BAYLOR, HURST, ALLEN, and BERTANI (1957) and are compatible with the findings of MCFALL and STENT (1958) that an anomaly of the genetic structure of a certain strain of T2H involves part of the *r*II region and at the same time is linked to *r*1. SEKELY (personal communication) has recently shown linkage between all known markers of T2L.

SUMMARY

This report describes sensitive tests of linkage, which have indicated that all the known markers of the T-even phages can be arranged in one linkage group. The results presented here agree with the independent findings of BAYLOR *et al.* (1957), who found a marker linked to both of previously unlinked groups in T2, and with those of MCFALL and STENT (1958), who described a genetic anomaly affecting markers on each of the remaining two groups, thus linking them.

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LOCUS DEPENDENCE OF THE PARAMUTANT R PHENOTYPE IN MAIZE[1]

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THE R^r gene in maize conditions anthocyanin pigmentation in the aleurone layer of the endosperm and in certain vegetative tissues of the plant. R^r gametes produced by heterozygotes containing either the stippled (R^{st}) or the marbled (R^{mb}) allele determine conspicuously reduced aleurone pigmentation and more weakly pigmented seedlings and anthers relative to the standard R^r phenotype (BRINK 1956; BRINK and WEYERS 1957; BRINK and MIKULA 1958). All, or nearly all, of the R^r gametes from these heterozygotes are so affected, and the change in R^r expression is heritable through successive generations. The term *paramutation* has been applied to these regularly occurring, directed, genetic changes (BRINK 1958a).

Data upon which it was concluded that the basis of paramutation is a change at, or near, the R locus, as opposed to an alteration in an autonomous cytoplasmic element affecting R^r expression, have been published previously by BRINK (1956, 1958a,b). The purpose of this paper is to present decisive evidence that the basis of paramutation is indeed chromosomal and that the immediate R region is involved. The new evidence shows that independently distinguishable R alleles of paramutant and nonparamutant origins, respectively, retain their identity in a common cytoplasm. Direct demonstration of R-locus dependence of the paramutant R phenotype was made possible by the use of a series of R^g mutants from standard R^r which were unchanged in aleurone pigment-producing action and in sensitivity to paramutation in heterozygotes with stippled, but which differed from R^r in giving green, rather than red, seedlings.

MATERIALS AND METHODS

The genetic background of the experimental stocks used was standardized by backcrossing the foundation strains four or more times to the inbred line, W22. An unrelated inbred, W23, was used as the $r^g r^g$ tester strain in most of the experiments. In these genetic backgrounds, standard R^r conditions self-colored aleurone in two or three doses in the triploid endosperm, and darkly mottled aleurone in single dose. In addition, R^r produces anthocyanin (red) in the seedling and anthers. The bottom recessive gene in the multiple allelic series, r^g, conditions colorless (no anthocyanin) aleurone and colorless (green) seedlings and anthers.

[1] Paper No. 767 from the Department of Genetics, College of Agriculture, University of Wisconsin, Madison 6, Wisconsin.

The allele r^r determines colorless aleurone and red seedlings and anthers. The paramutagenic alleles stippled (R^{st}) and marbled (R^{mb}) give fine and coarse spotting patterns of aleurone color on a colorless background, respectively, and no (or occasionally slight) anthocyanin in the seedlings and anthers.

The symbol R^g in this paper refers to mutant genes derived from standard R^r differing in plant, but not aleurone, color determination. A series of ten such mutants, designated R^g_1 to R^g_{10}, were isolated as colorless (green) seedlings among red sibs in the progeny of the cross W22 $R^rR^r \times$ W22 r^gr^g. For convenience and clarity in the text, R^r and R^g alleles extracted from heterozygotes containing R^{st} are designated $R^{r'}$ and $R^{g'}$, and those extracted from heterozygotes containing R^{mb} are designated $R^{r'''}$ and $R^{g'''}$, respectively.

The aleurone pigmenting capacities of the various alleles were measured in single dose in the triploid endosperm. This was done by using plants containing the alleles to be tested as pollen parents in crosses with either W23 r^gr^g or W22 r^gr^g. Individual testcross kernels were scored for pigmentation by matching them against a set of six standard kernels selected so as to define seven classes ranging from colorless (class 1) through increasing grades of mottling (classes 2 to 6) to self-colored (class 7). In tests of heterozygous plants carrying R alleles differing in plant color expression (for example, R^rR^g plants), the two genotypic classes of testcross kernels were separated following scoring for aleurone pigmentation by germinating the kernels and observing seedling color. Unless otherwise indicated, 100 kernels from each testcross ear were scored for aleurone color, and the aleurone phenotypes of the two respective genotypic classes were then evaluated on the basis of the first 42 kernels scored of the appropriate seedling class.

The experimental results to be presented later are based on the results of the series of testcrosses itemized below. The symbol R^g, as employed in this schedule of matings, refers to all the mutants of this class derived from standard R^r. Eight such R^g factors of independent origin were systematically tested.

(1) W23 r^gr^g ♀ $\times$ R^rR^g ♂

This type of mating was made in order to compare the aleurone pigment-producing action of each of the R^g mutants from standard R^r with standard R^r itself, under conditions in which the background inheritance was equalized. Origin of the mutants from standard R^r in a W22 inbred stock insures that the factors close to the R locus also were alike throughout.

(2) W23 r^gr^g ♀ $\times$ R^rR^{st} ♂

(3) W23 r^gr^g ♀ $\times$ R^gR^{st} ♂

(4) W23 r^gr^g ♀ $\times$ $R^{r'}R^g$ ♂

The respective staminate parents in testcrosses (3) and (4) were sibs derived from $R^rR^{st} \times R^gr^g$ matings.

(5) W23 r^gr^g ♀ $\times$ $R^rR^{g'}$ ♂

The staminate parents in this case were the offspring from $R^gR^{st} \times R^rR^r$ matings.

Testcrosses (2) and (3), above, provide for a comparison of the paramutability of the respective R^g mutants with that of the parent R^r allele, in heterozygotes with stippled.

Items (4) and (5) represent key testcrosses in the series. Two kinds of R^rR^g heterozygotes are involved as staminate parents. In (4), R^g is derived from a stock culture, and R^r from an R^rR^{st} heterozygote. Origin of the two alleles is reversed in mating (5); the R^g allele present is derived from an R^gR^{st} heterozygote, whereas the R^r factor is from an R^rR^r stock culture. The prediction, on the hypothesis that the paramutant R phenotype is R-locus dependent, is that two about equally frequent classes of kernels will result from each of the testcrosses, darkly mottled and weakly mottled, and that the latter class, in each instance, will be of the seeding color, red or green, corresponding to the allele that has been passed through the heterozygote with stippled, and, thus, is in the paramutant form.

(6) W23 r^gr^g ♀ × R^rR'' ♂

The staminate parents in this instance were derived from $R^rR^r \times R^rR^{st}$ matings. The hypothesis under examination calls for the production of two about equally frequent classes of kernels, darkly mottled and lightly mottled. Both kinds of seeds, however, will give red seedlings on germination, and so are not separable with reference to origin by an independent criterion, as in matings (4) and (5).

(7) W23 r^gr^g ♀ × $R^gR^{r'''}$ ♂

The $R^gR^{r'''}$ plants involved in this test were derived from R^gr^r ♀ × R^rR^{mb} ♂ matings, and thus provide a means of checking directly whether the paramutant form of the R^r allele extracted from a marbled heterozygote maintains its identity when paired with a mutant R^g factor derived from a stock culture.

(8) W23 r^gr^g ♀ × $R^{g'''}R^r$ ♂

The $R^{g'''}R^r$ plants tested in this mating were derived from the cross R^rR^r ♀ × R^gR^{mb} ♂. Since the origins of the R alleles were reversed relative to the staminate parents in (7) the expectation on the basis of the hypothesis under test is that the relative pigmenting capacities of the two alleles will be reversed accordingly. Thus, two classes of testcross kernels are expected, and the lightly pigmented class should produce green seedlings (R^g).

The genotypic constitutions of the male parents employed were verified throughout by the kernel phenotypes on the ear borne by the male parent, anther color of these individuals, and the testcross ear phenotypes.

EXPERIMENTAL RESULTS

Testcross (1) r^sr^s ♀ × R^rR^s ♂: All the kernels from testcrosses involving R^rR^g plants carrying, respectively, nine of the ten R^g mutants derived from standard R^r displayed a darkly mottled phenotype. Table 1 shows the distributions of aleurone color scores for the $R^rr^gr^g$ and $R^gr^gr^g$ classes of kernels, as separated retroactively by the seedling color test. No significant differences between the two classes in level of aleurone pigment-producing action were disclosed by the t test.

Comparison of the results from testcrosses (1) r^sr^s ♀ × R^sR^r ♂ *(2)* r^sr^s ♀ × R^rR^{st} ♂, *and (3)* r^sr^s ♀ × R^sR^{st} ♂: Three groups of testcross results are brought together in Table 2. In the first place, the aleurone color data for eight mutant R^g alleles, derived from R^gR^r staminate parents and thus in standard form, are

TABLE 1

Frequency distributions and means of aleurone color scores for $R^rr^sr^s$ *and* $R^sr^sr^s$ *kernels on testcross ears from W23* r^sr^s ♀ × *W22* R^rR^s ♂ *matings. Each distribution and mean represents the pooled tests of four staminate plants*

Staminate parent family	Endosperm genotype	Aleurone color classes							Mean score	t value
		1	2	3	4	5	6	7		
J79	$R^rr^gr^g$	..	..	..	8	83	77	..	5.41	0.442
	$R^g_1r^gr^g$	..	..	..	5	84	77	2	5.45	
J80	$R^rr^gr^g$	..	..	..	17	83	67	1	5.31	1.617
	$R^g_2r^gr^g$	..	..	..	5	84	79	..	5.44	
J81	$R^rr^gr^g$	..	..	..	1	64	94	9	5.66	0.632
	$R^g_3r^gr^g$	..	..	..	..	51	116	1	5.70	
J82	$R^rr^gr^g$	..	..	..	6	97	64	1	5.36	2.507
	$R^g_4r^gr^g$	..	..	..	4	72	91	1	5.53	
J83	$R^rr^gr^g$	..	..	1	19	94	54	..	5.20	1.446
	$R^g_5r^gr^g$	..	..	..	27	99	42	..	5.09	
J84	$R^rr^gr^g$	..	..	..	2	67	94	5	5.61	2.729
	$R^g_6r^gr^g$	..	..	..	6	81	81	..	5.45	
J85	$R^rr^gr^g$	..	..	..	6	94	67	1	5.38	2.119
	$R^g_7r^gr^g$	..	..	..	..	80	87	1	5.53	
J86	$R^rr^gr^g$	..	..	..	6	58	101	3	5.60	0.717
	$R^g_8r^gr^g$	..	..	..	..	56	108	4	5.69	
J87	$R^rr^gr^g$	..	..	..	7	105	56	..	5.29	1.729
	$R^g_{10}r^gr^g$	..	..	..	5	95	68	..	5.38	

brought forward from Table 1. Entered directly above each of these eight arrays are the values for the $R^{g'}r^gr^g$ testcross kernels resulting from the corresponding r^gr^g ♀ × $R^gR^{s't}$ ♂ matings. The difference between the paired distributions in each case affords a measure of the paramutability of the respective R^g mutants in heterozygotes with stippled. The last line in the table presents the pooled results for the $R^{r'}r^gr^g$ kernels from testcrosses of ten $R^rR^{s't}$ plants on r^gr^g females. This entry, when compared with the corresponding data from the eight $R^gR^{s't}$ testcrosses, provides a basis for determining whether the mutation of R^r to

R^g results in a change in paramutability of the allele when measured in terms of aleurone pigment-producing action.

Since the $R^{st}r^gr^g$ and $R^{g'}r^gr^g$ kernels on the ears from testcross (3) could not be differentiated by the seedling test, they were separated visually on the basis of aleurone phenotype alone. The stippled phenotype is distinguished by sharply defined pigment spots of relatively uniform size, whereas paramutant *R* kernels characteristically display somewhat diffusely pigmented areas that are more irregular in extent. A potential bias arises from the fact that occasional kernels carrying a stippled gene are colorless, and all colorless kernels were classified as $R^{g'}r^gr^g$. The observation that the two groups of kernels separated in this way were

TABLE 2

Frequency distributions and means of aleurone color scores for $R^{g'}r^gr^g$ *kernels from W23* r^gr^g ♀ × *W22* R^gR^{st} ♂ *matings in comparison with those of* $R^gr^gr^g$ *kernels from W23* r^gr^g ♀ × *W22* R^rR^g ♂ *matings. The bottom line of the table gives the corresponding values for the* $R^{r'}r^gr^g$ *kernels from testcrosses of standard* R^r *heterozygous with* R^{st} *on W23* r^gr^g *females*

Staminate parent		No. plants tested	Aleurone color class							Mean score
Pedigree no.	Genotype		1	2	3	4	5	6	7	
J36	$R^g_1R^{st}$	4	12	105	44	7	..	..	..	2.27
J79	$R^g_1R^r$	4	..	..	..	5	84	77	2	5.45
J37	$R^g_3R^{st}$	4	4	97	37	26	4	..	..	2.58
J81	$R^g_3R^r$	4	..	..	..	..	51	116	1	5.70
J38	$R^g_4R^{st}$	4	4	18	109	37	..	..	..	3.07
J82	$R^g_4R^r$	4	..	..	..	4	72	91	1	5.53
J39	$R^g_5R^{st}$	4	33	130	5	..	..	..	..	1.83
J83	$R^g_5R^r$	4	..	..	..	27	99	42	..	5.09
J40	$R^g_6R^{st}$	4	1	61	101	4	1	..	..	2.66
J84	$R^g_6R^r$	4	..	..	..	6	81	81	..	5.45
J41	$R^g_7R^{st}$	4	8	89	57	14	..	..	..	2.46
J85	$R^g_7R^r$	4	..	..	..	..	80	87	1	5.53
J42	$R^g_8R^{st}$	4	9	120	36	3	..	..	..	2.20
J86	$R^g_8R^r$	4	..	..	..	..	56	108	4	5.69
J43	$R^g_{10}R^{st}$	4	4	64	82	18	..	..	..	2.68
J87	$R^g_{10}R^r$	4	..	..	..	5	95	68	..	5.38
J35	R^rR^{st}	10	3	176	218	21	2	..	..	2.63

approximately equal in number indicates, however, that this source of error is not important.

The data in Table 2 show that all, or nearly all, of the $R^{g\prime}$ gametes produced by the eight groups of R^gR^{st} heterozygotes are markedly impaired in pigmenting potential. The paramutant and nonparamutant kernel classes overlap little or not at all in each of the paired distributions. This relation is even more evident when it is considered that the scoring method did not differentiate between kernels with phenotypes at opposite extremes of a single pigment class and, consequently, tends slightly to exaggerate the overlap.

The average pigmenting capacity of $R^{g\prime}$ gametes from each set of R^gR^{st} plants appears similar to that of $R^{r\prime}$ gametes from R^rR^{st} plants. The average scores for the eight groups of $R^{g\prime}r^gr^g$ kernels in Table 2 is 2.47. This value is close to the mean score for the $R^{r\prime}r^gr^g$ kernels, which was 2.63.

Testcrosses (4) r^gr^g ♀ × $R^{r\prime}R^g$ ♂ *and (5)* r^gr^g ♀ × $R^rR^{g\prime}$ ♂: The experimental results just presented establish two facts that make the tests for locus dependence of the paramutant R phenotype, now to be discussed, decisive. The mutant R^g alleles derived from standard R^r are indistinguishable from the latter in (a) aleurone pigment-producing action when in standard form, and (b) paramutability in heterozygotes with stippled. Added to equivalence in these two essential respects is the further characteristic, upon which design of the experiments in question rests, that R^gr^g kernels yield green seedlings, whereas R^rr^g kernels give red seedlings, thus permitting definitive separation of the two classes of seeds in segregating populations.

Testcrosses (4) and (5) involved R^rR^g plants as staminate parents, in which one, but not the other, of the two R alleles was derived from a stippled heterozygote in the previous generation, and thus was in the paramutant form. In testcross (4) it is the R^r allele that has this history, whereas in testcross (5), it is one or another of the mutant R^g factors that is derived from an R^gR^{st} parent. The source of the other allele in the heterozygotes, in each case, was a stock culture.

The results expected from such testcrosses differ according to the assumptions made regarding the basis of the differences between the paramutant and nonparamutant phenotypes. If paramutation does, in fact, involve a chromosomal change at or near the R locus, it is expected that two equally frequent phenotypic classes of testcross kernels would be observed, a lightly pigmented paramutant class carrying an R allele derived from a stippled heterozygote in the previous generation, and a more darkly pigmented class containing the nonparamutant form of R characteristic of R R stock cultures. On the other hand, if the basis of paramutation is a regularly transmissible cytoplasmic element, the two classes of testcross kernels containing R genes of paramutant origin and those containing R genes of nonparamutant origin should display the same phenotype, because, as previously shown, R^r and R^g normally are equivalent in aleurone pigment-producing action, and they react alike to the conditions under which the paramutant phenotype arises.

The data from testcrosses of the type r^gr^g ♀ × $R^{r\prime}R^g$ ♂ are summarized in Table 3. Eight R^g mutants from standard R^r were involved in the matings. Since these

TABLE 3

Frequency distributions and means of aleurone color scores for $R^{r'}r^{g}r^{g}$ *and* $R^{g}r^{g}r^{g}$ *kernels from the cross W23* $r^{g}r^{g}$ ♀ × *W22* $R^{r'}R^{g}$ ♂ (*ex.* $R^{r}R^{st} \times R^{g}r^{g}$). *Each distribution and mean represents the pooled tests of ten staminate plants*

Staminate parent family	Endosperm genotype	Aleurone color classes							Mean score	t value
		1	2	3	4	5	6	7		
J36	$R^{g}r^{g}r^{g}$ 1	1	8	9	251	123	28	..	4.36	19.06**
	$R^{r'}r^{g}r^{g}$	50	317	41	7	4	1	..	2.05	
J37	$R^{g}r^{g}r^{g}$ 3	6	1	3	107	214	89	..	4.88	12.95**
	$R^{r'}r^{g}r^{g}$	1	22	132	243	19	3	..	3.63	
J38	$R^{g}r^{g}r^{g}$ 4	6	..	3	104	249	57	1	4.82	17.50**
	$R^{r'}r^{g}r^{g}$	5	75	240	94	5	1	..	3.05	
J39	$R^{g}r^{g}r^{g}$ 5	8	8	33	266	91	14	..	4.11	13.72**
	$R^{r'}r^{g}r^{g}$	103	305	10	1	..	1	..	1.79	
J40	$R^{g}r^{g}r^{g}$ 6	1	1	2	99	244	73	..	4.91	13.89**
	$R^{r'}r^{g}r^{g}$	..	24	169	214	10	3	..	3.52	
J41	$R^{g}r^{g}r^{g}$ 7	1	1	4	129	225	60	..	4.80	11.27**
	$R^{r'}r^{g}r^{g}$	2	40	162	208	8	..	..	3.43	
J42	$R^{g}r^{g}r^{g}$ 8	1	10	3	186	183	37	..	4.55	15.94**
	$R^{r'}r^{g}r^{g}$	4	305	103	4	4	..	..	2.28	
J43	$R^{g}r^{g}r^{g}$ 10	2	..	2	79	238	99	..	5.02	10.82**
	$R^{r'}r^{g}r^{g}$	..	26	144	240	8	2	..	3.56	

** Significant at the 1 percent level.

mutants, although of independent origin, are indistinguishable in action, the experiment, in effect, is an eight-fold repetition of the same test. It will be seen from the table that the testcross kernels yielding green seedlings (R^{g}) show higher mean scores for aleurone color throughout than do their counterparts giving red seedlings (R^{r}). The difference is large in each case, and is very highly significant statistically. Thus, the capacity for reduced aleurone pigmentation of the testcross kernels regularly follows the allele descended from the stippled heterozygote —in this case, the R^{r} factor.

The results of the testcrosses in which the sources of the R^{r} and R^{g} alleles in the staminate parents were reversed are shown in Table 4. The kernel scores which appear in Table 4 should not be compared directly with those in Table 3 because the testcrosses were made in different years, and somewhat different sets of standard kernels were used in scoring in the two cases. These circumstances explain the higher average scores in Table 4. The data reveal that, throughout this series, the testcross kernels giving red seedlings (R^{r}) gave very significantly higher mean

TABLE 4

Frequency distributions and means of aleurone color scores for $R^{g'}r^{g}r^{g}$ *and* $R^{r}r^{g}r^{g}$ *kernels from crosses of the type W23* $r^{g}r^{g}$ ♀ × *W22* $R^{r}R^{g'}$ ♂ *(ex.* $R^{g}R^{st}$ × $R^{r}R^{r}$)

Staminate parent	No. plants tested	Endosperm genotype	Aleurone color classes							Mean score	t value
			1	2	3	4	5	6	7		
D35	14	$R^{g'}_{1}r^{g}r^{g}$	4	1	23	332	200	27	1	4.37	14.00**
		$R^{r}r^{g}r^{g}$	2	..	1	25	156	404	..	5.63	
D36	15	$R^{g'}_{3}r^{g}r^{g}$	9	281	162	126	39	13	..	2.90	15.31**
		$R^{r}r^{g}r^{g}$	2	3	4	60	240	321	..	5.38	
D37	14	$R^{g'}_{4}r^{g}r^{g}$	..	15	58	399	102	14	..	4.07	19.70**
		$R^{r}r^{g}r^{g}$	2	2	1	40	195	348	..	5.50	
D38	15	$R^{g'}_{5}r^{g}r^{g}$	3	166	206	223	23	9	..	3.20	34.95**
		$R^{r}r^{g}r^{g}$	..	5	9	33	203	380	..	5.50	
D39	15	$R^{g'}_{6}r^{g}r^{g}$	1	2	..	193	333	101	..	4.84	12.54**
		$R^{r}r^{g}r^{g}$	2	..	..	22	205	400	1	5.59	
D40	15	$R^{g'}_{7}r^{g}r^{g}$	3	7	33	349	196	42	..	4.36	10.83**
		$R^{r}r^{g}r^{g}$	3	..	..	26	187	413	1	5.60	
D41	15	$R^{g'}_{8}r^{g}r^{g}$	3	20	53	353	174	27	..	4.20	10.75**
		$R^{r}r^{g}r^{g}$	3	..	..	22	173	432	..	5.63	
D42	13	$R^{g'}_{10}r^{g}r^{g}$	5	36	122	316	58	9	..	3.76	14.53**
		$R^{r}r^{g}r^{g}$	1	1	4	23	145	372	..	5.61	

** Significant at the 1 percent level.

scores for aleurone color than the kernels yielding green seedlings (R^{g}). Again, therefore, the capacity for reduced aleurone pigmentation follows the particular factor previously carried in a stippled heterozygote—in this instance, R^{g}.

It is apparent that, whereas R^{r} and R^{g} initially are equivalent in aleurone pigment-producing action and yield the same paramutant phenotype, they may be differentiated from each other regularly by passing either one through a heterozygote with stippled. Whatever the means by which the paramutant phenotype is mediated during development of the plant, these results show that the phenomenon rests upon a distinctive chromosomal condition at, or near, the *R* locus.

The reversal of the relative pigmenting capacities of the R^{r} and R^{g} alleles, which results from reversal in the origins of the two alleles, is illustrated in the photograph in Figure 1, and the frequency polygons in Figure 2.

Testcross (6) $r^{g}r^{g}$ ♀ × $R^{r}R^{r'}$ ♂: One hundred thirty kernels from each testcross ear were scored by the kernel matching procedure in this group of material. The frequency distributions for aleurone color are entered in Table 5 and are represented graphically in Figure 3. It will be noted that the distributions are bimodal

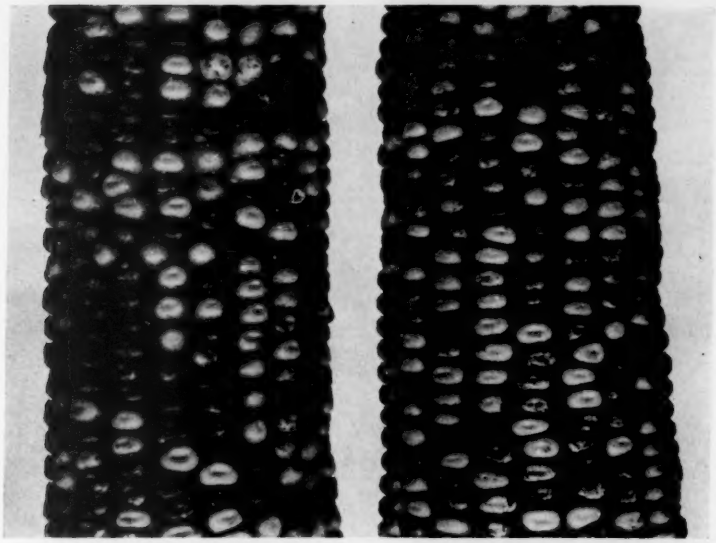

FIGURE 1.—Mendelian distributions of darkly mottled and weakly pigmented kernels on testcross ears from two different $r^g r^g$ ♀ × $R^g_s R^r$ ♂ matings, proving that the paramutant aleurone phenotype is *R*-locus dependent. Left: R^g_s allele was derived from an $R^g_s R^{st}$ heterozygote, and R^r came from an $R^r R^r$ stock culture. The 50 percent of weakly pigmented kernels, on sprouting, yielded green seedlings ($R^{g'}_s$), and the darkly colored kernels gave red seedlings (R^r). Right: The R^g_s was derived from a stock $R^g_s R^g_s$ culture, and R^r came from an $R^r R^{st}$ heterozygote. The 50 percent weakly pigmented kernels, in this instance, gave red seedlings ($R^{r'}$), whereas the darker kernels yielded green seedlings (R^g_s). (Reproduced from the *Quarterly Review of Biology* with permission of the Editor).

TABLE 5

Frequency distributions for $R^r r^g r^g$ *and* $R^{r'} r^g r^g$ *kernels on individual ears from crosses of W23* $r^g r^g$ *female with* $R^{r'} R^r$ *male (ex.* $R^r R^{st} \times R^r R^r$)

Mating	Distribution of aleurone color scores						
	1	2	3	4	5	6	7
W23 × 45-730-3	..	5	40	31	53	1	..
-4	..	7	44	34	42	3	..
-6	..	2	43	31	48	6	..
-7	..	19	43	10	55	3	..
-8	..	6	46	16	60	2	..
-9	..	7	46	20	53	4	..
-10	..	..	41	35	50	4	..
-12	..	7	54	25	37	7	..

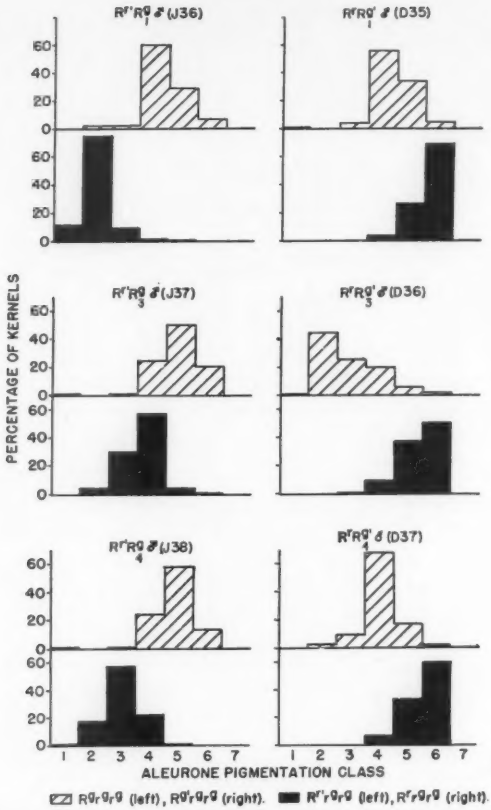

FIGURE 2.—Percentages of kernels from the test matings $r^g r^g$ ♀ × $R^r R^g$ ♂ (left) and $r^g r^g$ ♀ × $R^r R^{g'}$ ♂ (right) in seven pigmentation classes. The staminate testcross parents are indicated above the histograms.

throughout, with the largest numbers of entries in the "3" and "5" color classes. This is the result expected if, in the $R^r R^{r'}$ staminate parent, the R^r allele derived from an $R^r R^r$ stock culture is strong, and the $R^{r'}$ allele, extracted from an $R^r R^{st}$ heterozygote, is relatively weak, in aleurone pigment-producing action. Since both R^r and $R^{r'}$ condition red seedlings and their aleurone color effects overlap, the available data do not prove that this is the basis of the bimodality of the frequency distributions. By analogy with the results presented in the preceding section it may be inferred, however, that the reduction in pigment-producing action which R^r is known to undergo in $R^r R^{st}$ heterozygotes tends strongly to be maintained in the $R^r R^{r'}$ offspring from $R^r R^{st}$ × $R^r R^r$ matings.

Testcrosses (7) $r^g r^g$ ♀ × $R^g R^{r'''}$ ♂ *and (8)* $r^g r^g$ ♀ × $R^{g'''} R^r$ ♂: Confirmatory

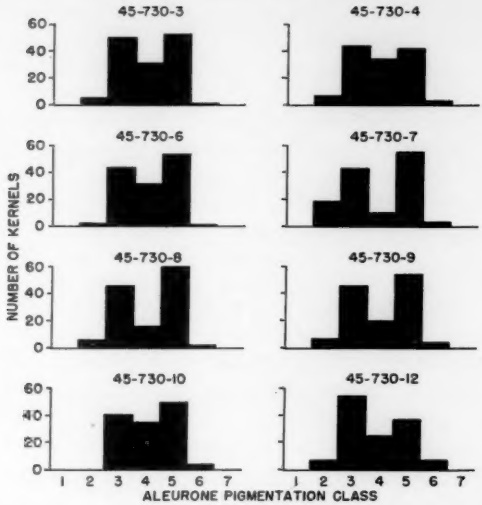

FIGURE 3.—Frequencies of $R^{r}r^{g}r^{g}$ and $R^{r'}r^{g}r^{g}$ testcross kernels in seven pigmentation classes. The staminate testcross parents are indicated above the histograms.

TABLE 6

Mean aleurone color scores for $R^{r}r^{g}r^{g}$ *and* $R^{g}r^{g}r^{g}$ *kernels from the crosses* $r^{g}r^{g}$ ♀ × $R^{r'''}R^{g}$ ♂ (*ex.* $R^{g}r^{r} \times R^{r}R^{mb}$) *and* $r^{g}r^{g}$ ♀ × $R^{r}R^{g'''}$ ♂ (*ex.* $R^{r}R^{r} \times R^{g}R^{mb}$)

Mating	No. of ears	Mean aleurone color scores $R^{r}r^{g}r^{g}$ kernels	$R^{g}r^{g}r^{g}$ kernels
$r^{g}r^{g}$ ♀ × $R^{r'''}R^{g}$ ♂ (ex. $R^{g}r^{r} \times R^{r}R^{mb}$)			
W22 × W535	4	4.56	6.25
× W536	4	4.23	6.03
× W537	4	4.14	6.15
$r^{g}r^{g}$ ♀ × $R^{r}R^{g'''}$ ♂ (ex. $R^{r}R^{r} \times R^{g}R^{mb}$)			
W22 × W505 (c)	6	6.27	4.33

evidence for *R*-locus dependence of the *R* paramutant phenotype was obtained from experiments in which the paramutant alleles, $R^{r'''}$, or $R^{g'''}$, were derived from heterozygotes with the paramutagenic marbled (R^{mb}) factor. The reversal plan described under testcrosses (4) and (5) was again used, whereby R^{r} or R^{g} was passed through an R^{mb} heterozygote and then tested for pigment-producing action in plants carrying the contrasting allele (either R^{g} or R^{r}) derived from a stock culture.

It is evident from the results summarized in Table 6 that in both the $R^{r}R^{g'''}$ and $R^{r'''}R^{g}$ groups of plants tested in matings on W22 $r^{g}r^{g}$ ♀♀, the lower mean score for aleurone color was regularly associated with the allele, either R^{r} or R^{g}, that had been passed through the R^{mb} heterozygote. Thus, the genetic change

engendered in the heterozygote by marbled, as in the case of stippled also, is intrachromosomal, and subsequently follows the *R* locus in inheritance.

Table 7 is designed to bring together in convenient form the data from Tables 3 and 4 to which particular reference will be made in the Discussion.

Secondary processes influencing R *action:* Two secondary processes are known to be operative in $R^rR^{g\prime}$ and $R^{r\prime}R^g$ plants which tend to moderate the distinctive effects of these pairs of alleles on aleurone pigmentation in testcrosses on r^gr^g ♀♀. The first of these processes has been termed reversion (BRINK 1958a). Following extraction in paramutant form from R^rR^{st} or R^rR^{mb} plants, $R^{r\prime}$ reverts partially toward the level of pigment-producing action characteristics of standard R^r. No genotype has yet been found (aside from those carrying R^{st}, R^{mb}, or other paramutagenic allele) in which such partial reversion does not occur. The amount of reversion is variable, but in no established case has the full action of standard R^r been regained.

The other secondary process was first noted during the course of the present investigation, and involves an aspect of paramutation that will be dealt with in detail in a subsequent paper. It is found that in R^rR^{st} plants not only is the R^r factor reduced in pigment-producing potential but it also becomes paramutagenic, although only weakly so, as compared with R^{st}.

Both these processes would be expected to reduce the difference in aleurone pigment-producing potential of the paired factors in $R^rR^{g\prime}$ and $R^{r\prime}R^g$ plants. Partial reversion would result in shifts toward the standard level of action of the paramutant $R^{g\prime}$and $R^{r\prime}$ alleles; and the secondary paramutation promoted by $R^{g\prime}$ and $R^{r\prime}$ would weaken somewhat the effect on aleurone color of their respective R^r and R^g mates. Since the two processes are convergent in their effects, there would be a tendency for both alleles in $R^rR^{g\prime}$ and $R^{r\prime}R^g$ heterozygotes to change in the direction of a common level of pigment-producing action.

The significance of these facts in the present connection is that, although the secondary processes in question are operative in testcrosses on r^gr^g ♀♀ of $R^rR^{g\prime}$

TABLE 7

Mean aleurone color scores for $R^rr^gr^g$ *and* $R^gr^gr^g$ *kernels from outcrosses of W22* $R^{r\prime}R^g$ *males (ex.* $R^rR^{st} \times R^gr^g$) *and W22* $R^rR^{g\prime}$ *males (ex.* $R^rR^{st} \times R^rR^r$) *on W23* r^gr^g *females*

R^g allele	$R^{r\prime}R^g$ staminate parent		$R^rR^{g\prime}$ staminate parent	
	$R^{r\prime}r^gr^g$	$R^gr^gr^g$	$R^{r\prime}r^gr^g$	$R^{g\prime}r^gr^g$
(1)	(2)	(3)	(4)	(5)
R^g_1	2.05	4.36	5.63	4.37
R^g_3	3.63	4.88	5.38	2.90
R^g_4	3.05	4.82	5.50	4.07
R^g_5	1.79	4.11	5.50	3.20
R^g_6	3.52	4.91	5.59	4.84
R^g_7	3.43	4.80	5.60	4.36
R^g_8	2.28	4.55	5.63	4.20
R^g_{10}	3.56	5.02	5.61	3.76

and $R^{r'}R^{g}$ plants, they do not override the basic difference in aleurone color determining action of the paired factors in either case. Or, to state the point in another way, the residual difference between the two alleles derived from $R^{r}R^{g'}$ and $R^{r'}R^{g}$ heterozygotes is still large enough to be readily demonstrable in testcrosses on $r^{g}r^{g}$ ♀♀, as the data in Tables 3 and 4 show. There is little doubt, however, that much of the overlapping in the aleurone color scores of the "red seedling" and "green seedling" classes of testcross kernels in such cases is attributable to partial reversion of the paramutant allele plus secondary paramutation of the other factor.

DISCUSSION

The initial observations on the reduced pigment-producing action of the R^{r} allele in maize following passage of R^{r} through a heterozygote with stippled created a strong presumption in favor of the view that the genetic basis of the phenotypic change was intrachromosomal (BRINK 1956). An explanation of the phenomenon in terms of a self-reproducing cytoplasmic element appeared improbable on the grounds that both male and female R^{r} gametes produced by $R^{r}R^{st}$ plants invariably determined weakly colored kernels, when appropriately tested. Pollen transmission of a plasmid had not been observed in maize in any previous instance; and in no other Angiosperm had it been found that even a majority, not to say all, of the offspring could acquire an element of this kind from a potential donor through the male gametophyte. Furthermore, it was demonstrated that color of the $R^{r}R^{st}R^{st}$ kernels from $R^{st}R^{st}$ ♀ × $R^{r}R^{r}$ ♂ crosses was not depressed below the level expected to result from the action of normally functioning R^{st} and R^{r} alleles, even though $R^{r'}R^{st}R^{st}$ seeds from $R^{st}R^{st}$ ♀ × $R^{r}R^{st}$ ♂ matings clearly showed reduced pigmentation. This fact precluded stippled cytoplasm, *per se*, as the basis of the paramutant R^{r} phenotype.

Positive evidence to exclude an autonomous cytoplasmic element as the genetic basis of the phenomenon was obtained from the following experiment (BRINK 1958a). $R^{r}R^{r}$ plants were pollinated by $R^{st}r^{r}$ individuals, and the resulting two classes of offspring, $R^{r}R^{st}$ and $R^{r}r^{r}$, were then testcrossed on $r^{r}r^{r}$ ♀♀. The R^{r} gametes from the $R^{r}R^{st}$ plants only were found to determine the paramutant phenotype. The R^{r} gametes produced by the $R^{r}r^{r}$ sibs proved to be unchanged in aleurone color potential. This, of course, is not the result expected if an autonomous, pollen transmissible, cytoplasmic element conditions the reduced color expression of R^{r}.

Testcrosses on $r^{g}r^{g}$ ♀♀ of $R^{r}R^{r'}$ plants, derived from $R^{r}R^{st}$ × $R^{r}R^{r}$ matings, had disclosed also that two about equally frequent classes of kernels differing in grade of aleurone mottling often resulted. These seemingly Mendelian distributions were in accord with the view that the paramutant phenotype was *R*-locus dependent. There were two reasons, however, for considering this evidence less than decisive. The presumed R^{r} and $R^{r'}$ progeny of the $R^{r}R^{r'}$ plants were not distinguishable by an independent criterion, because the chromosomes concerned did not carry the necessary markers. Secondly, although there was clear evidence of bimodality throughout in the kernel populations resulting from $r^{g}r^{g}$ ♀ × $R^{r}R^{r'}$

matings in terms of aleurone color grade, the amount of overlap between the two presumed phenotypes was great enough on occasional ears to render an estimate of relative frequencies meaningless. Reversion of $R^{r'}$ toward the level of action characteristic of standard R^r, in $R^rR^{r'}$ plants, was recognized as a factor probably contributing to the overlap in the R^r and $R^{r'}$ phenotypes; but this circumstance alone appeared insufficient to account for the intergradation in color intensity among the kernels on testcross ears showing the greatest overlap. It was not known at the time the first experiments with $R^rR^{r'}$ plants were made that a second factor, namely, paramutagenicity of $R^{r'}$, also was operative in $R^rR^{r'}$ heterozygotes in reducing the difference in pigment-producing potential of the two alleles. Until paramutation at this secondary level was established, it was not possible to explain satisfactorily why about 50 percent of the kernels resulting from r^gr^g ♀ × $R^rR^{r'}$ ♂ testcrosses were not of the rather uniform darkly mottled phenotype obtained in r^gr^g ♀ × standard R^rR^r ♂ matings, and so distinguishable, in most instances at least, from the equally frequent $R^{r'}r^gr^g$ sib seeds, even after allowance was made for partial reversion of $R^{r'}$ toward the R^r level of action.

The foregoing considerations made it apparent that a critical test of the hypothesis that, in an *R R* plant derived from an $R\ R^{st}$ × *R R* cross the *R* allele with a history of heterozygosity for stippled in the preceding generation is weaker in aleurone pigment-producing potential than its *R* mate derived from a stock culture requires, in the first instance, that the two respective *R* factors be identifiable in the progeny as to origin. This condition was met in the present experiments by the use of a series of R^g mutants from W22 standard R^r, distinguishable with certainty in the early seedling stage by their differential effect on anthocyanin pigmentation. All the R^g mutants used were found to be indistinguishable in standard form from R^r in aleurone pigmentation, and all proved to be highly paramutable in heterozygotes with stippled (R^gR^{st}).

Two minor sources of error attending the use of such R^g factors in r^gr^g ♀ × R^rR^g ♂ testcrosses should be mentioned. R^r mutates to R^g in the W22 stock at a rate of about 3.0×10^{-4}. Obviously such rarely occurring mutations can be ignored in the present context. The second possible source of error is heterofertilization. A previous test with the W22 inbred strain disclosed 33 cases among 1995 offspring from r^gr^g ♀ × R^rr^g ♂ matings in which aleurone and seedling color were reversed in terms of the R^r and r^g alleles present in the staminate parent. Seventeen kernels with colored aleurone were found that gave green seedlings, and 16 kernels with colorless aleurone that gave red seedlings. Most, or all, of these exceptions are due to the functioning of two, rather than one, pollen tubes, carrying unlike male gametes, in fertilization of egg and central cell of a given female gametophyte. The frequency of such heterofertilization in this case is 1.7 percent, and would be expected to give an error of corresponding size in classification of the offspring from the r^gr^g ♀ × R^rR^g ♂ matings employed in the present study. Heterofertilization, however, appears not to be directed in any sense, so that the discrepancies arising from this source with respect to individual kernels, when a heterozygote is used as the staminate parent in a testcross, tend to cancel out each other in the difference between mean scores for alternative classes of seeds.

The principal body of new experimental data relating to *R*-locus dependence of the paramutant phenotype are summarized in Table 7. Reference to columns 2 and 3 in the table shows that following $r^g r^g$ ♀ × $R^{r'}R^g$ ♂ testcrosses, involving eight R^g mutants from R^r, the kernels yielding red seedlings are more weakly pigmented, on the average, than those giving green seedlings. As previously stated, the difference is statistically significant in each of the eight comparisons. Thus the R^r allele, on being passed through a heterozygote with R^{st} in the previous generation, and then paired with R^g from a stock culture, in an $R^{r'}R^g$ plant, is reduced in aleurone pigment-producing potential, relative to R^g, to which it is equivalent in this respect when in standard form.

The results obtained when the origin of R^r and R^g are reversed, as between a heterozygous stippled parent and a stock culture, are shown in columns 4 and 5 in Table 7. The 50 percent of kernels which yielded green seedlings were found to be significantly inferior in pigmentation, in each of the eight comparisons, in this case, to those giving red seedlings. Again, therefore, it is the allele in the R^rR^g staminate parent derived from the heterozygote with stippled that is the weaker in pigment-producing action in the testcross kernels.

It is evident from these data that reversing the origin of R^r and R^g as between a stock culture plant and a stippled heterozygote correspondingly reverses the order in which R^r and R^g stand in level of aleurone pigment determination. R^r and R^g as carried in stock cultures, are equivalent in aleurone pigment-producing action. Both are changed to weakly acting, paramutant forms in heterozygotes with stippled. The testcross data reviewed show that, following such change, each persists through the next generation in differentiated form in the common cytoplasm of a plant carrying, as a control, the oppositely marked factor, either R^r or R^g, derived from a stock culture. The results of parallel tests with paramutant R^r and R^g alleles derived from heterozygotes with marbled are in agreement with these findings. Partial reversion of the paramutant allele toward the normal form and secondary paramutation of the normal allele, in $R^{r'}R^g$ and $R^rR^{g'}$ heterozygotes, reduces but does not obliterate the difference in action between the paired factors.

It is noteworthy that partial reversion and secondary paramutation, operating at somewhat more intensive levels than those encountered, could have rendered impossible the demonstration of *R* locus dependence of the paramutant phenotype by the procedure followed in the present experiments.

SUMMARY

Conclusive evidence for the *R*-locus dependence of the paramutant *R* phenotype was obtained by showing that independently distinguishable *R* alleles of paramutant and nonparamutant origins, respectively, retain their identity in a common cytoplasm. This was made possible by the use of a series of R^g mutants from standard R^r which were found to be equivalent to the latter in aleurone pigmenting capacity and in paramutability in heterozygotes with R^{st}, but which were distinguishable from R^r in conditioning green rather than red seedlings.

$R^{r'}R^{g}$ and $R^{r}R^{g'}$ plants representing eight independent R^{g} mutants were constructed from matings of the types $R^{r}R^{st}$ ♀ × $R^{g}r^{g}$ ♂ and $R^{g}R^{st}$ ♀ × $R^{r}R^{r}$ ♂, respectively, and testcrossed on $r^{g}r^{g}$ ♀♀. The testcross kernels were scored by a kernel matching procedure, and then separated definitively into $R^{r'}r^{g}r^{g}$ and $R^{g}r^{g}r^{g}$ or $R^{g'}r^{g}r^{g}$ and $R^{r}r^{g}r^{g}$ classes by the seedling test. The ability to separate $R^{r}r^{g}$ and $R^{g}r^{g}$ sibs on the basis of seedling color was an essential feature in the design of the experiments. The results showed that the paramutant phenotype followed the allele previously paired in heterozygotes with R^{st}, and the more darkly pigmented phenotype followed the allele derived from a stock culture. When $R^{r'}R^{r}$ plants from the mating $R^{r}R^{st}$ × $R^{r}R^{r}$ were testcrossed, a bimodal distribution of aleurone phenotypes resulted, in agreement with the expectation on the basis of *R*-locus dependence of paramutation. The results obtained when R^{mb} was substituted for R^{st} as the paramutagenic allele in heterozygotes with R^{r} or R^{g} were confirmatory.

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